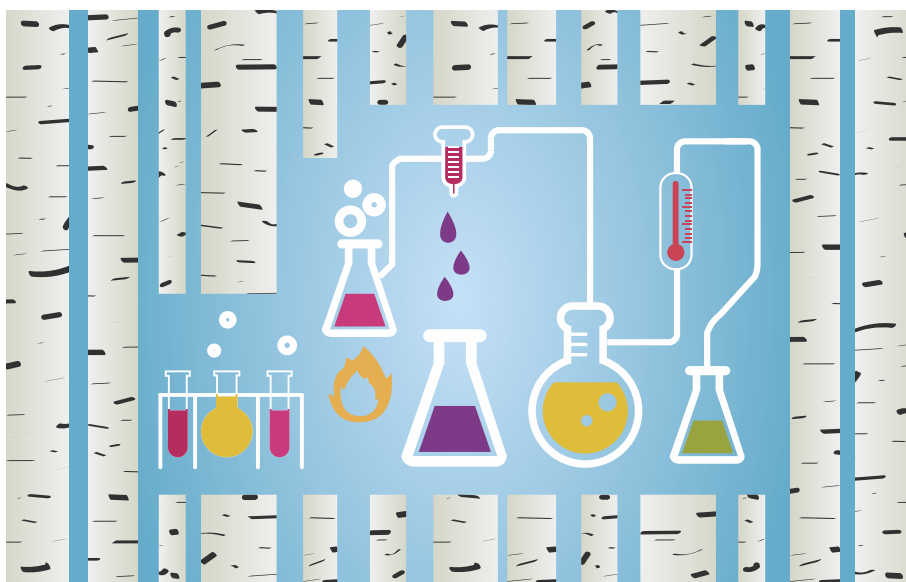




**RAISA HAAVIKKO**

## **Synthesis of Betulin Derivatives with New Bioactivities**



DIVISION OF PHARMACEUTICAL CHEMISTRY AND TECHNOLOGY  
FACULTY OF PHARMACY  
DOCTORAL PROGRAMME IN DRUG RESEARCH  
UNIVERSITY OF HELSINKI

Division of Pharmaceutical Chemistry and Technology  
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University of Helsinki  
Finland

# **Synthesis of betulin derivatives with new bioactivities**

Raisa Haavikko

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy of the University of Helsinki, for public examination in Auditorium 1041, Viikki Biocenter 2, on November 6<sup>th</sup> 2015, at 12 noon.

Helsinki 2015

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ISBN 978-951-51-1617-8 (nid.)  
ISBN 978-951-51-1618-5 (PDF)  
ISSN 2342-3161 (Print)  
ISSN 2342-317X (Online)

Cover science laboratory illustration designed by Freepik.com  
Cover image by Hape Haavikko  
Hansaprint Printing House  
Helsinki 2015

*"I am always doing that which I cannot do, in order that I may learn how to do it."*  
*Pablo Picasso*

## Abstract

Betulin is a triterpene class natural product present, for example, in the outer layer of birch bark, which is a low-value waste product of the forestry industry in Northern Hemisphere. Oxidation products of betulin, namely betulinic acid and betulonic acid, have been shown to have several biological activities. This makes betulin an interesting starting point for drug discovery projects.

In this work, mainly ring A-fused heterocyclic derivatives of betulin were synthesized, and their structure-activity relationships against a protozoan parasite *Leishmania donovani*, prostate cancer cell lines, a serine hydrolase enzyme (ABHD12), and inflammatory factors were studied. The heterocycles used included pyridine, pyrazine, pyrazole, indole, isoxazole, and oxazole rings. Also, positions C-28 and C-20 of these compounds were modified and the resulting structure-activity relationships (SAR) were studied.

Among the tested compounds, two heterocyclic betulin derivatives showed significant inhibition against *L. donovani* amastigotes growth. These compounds showed improved activity compared to the heterocyclic betulin derivatives tested previously. Also, a betulin-derived potent anti-HIV compound, bevirimat, showed *L. donovani* growth inhibition at a similar level to the ring A-fused heterocyclic betulin derivatives. However, further optimization is needed to get more potent betulin derivative activity against *L. donovani*.

In prostate cancer studies some of the betulin derivatives displayed dose-dependent anti-invasive activity at nanomolar concentrations with negligible cytotoxicity. The most potent compounds were betulin derivatives with a ring A-fused heterocycle. Also, the carboxyl group at C-28 seemed to be important for activity. These compounds showed considerably improved anti-invasive effects compared to betulinic acid.

Betulin derivatives were found to selectively inhibit the ABHD12 serine hydrolase enzyme without inhibiting other endocannabinoid hydrolases and without activity towards cannabinoid receptors. In mechanistic studies, the inhibition type was shown to be reversible. Important structural features required for ABHD12 inhibition were revealed based on our SAR studies.

Heterocyclic betulin derivatives were shown to suppress the expression of several inflammation mediators, such as iNOS, IL-6, and MCP-1, a pyrazole derivative being the most potent compound. With further improvement and development, more potent betulin derivatives could be found.

The betulin derivatives show different activity with different targets, which means they are selective. Selectivity and potency could be further improved to obtain a potential betulin-derived compound for further development. In this work, betulin has been shown to be a good starting point for several different drug discovery projects.

## Acknowledgements

This work was carried out at the Division of Pharmaceutical Chemistry and Technology at the Faculty of Pharmacy, University of Helsinki during the years 2009-2015. Funding by Finnish Cultural Foundation and University of Helsinki is gratefully acknowledged.

I am deeply thankful to my supervisor Prof. Jari Yli-Kauhaluoma for making this work possible. You have always been available for discussions, no matter how small or big the issue has been. Your enthusiasm is really catching, and has encouraged me when I have been in doubt. I am grateful to my other supervisor Dr. Vânia M. Moreira, for her help in writing the manuscripts and this thesis.

I want to thank all my collaborators and co-authors. Without you, this work would not have been possible! Especially I am grateful to Dr. Ville Härmä, Dr. Matthias Nees, Dr. Kirsi-Marja Oksman-Caldentey, Mirka Laavola, Prof. Eeva Moilanen, Dr Teija Parkkari, Doc. Jarmo T. Laitinen, Prof. Charles L. Jaffe and his group for your efforts towards publications this thesis is based on. Additionally, I want to acknowledge Professor Leonardo Scapozza and Assistant Professor Samuel Silvestre for their thorough review and valuable comments.

I wish to thank all the colleagues at the former Division of Pharmaceutical Chemistry. You have made work here enjoyable. Without our relaxed atmosphere and regular coffee breaks this would have been just all work without joy. Specifically I would like to thank Leena and Titti for your friendship and all the help you have given, personally and professionally. Thanks to Kristian for sharing an office with me for years, and all the IT help you have provided. I would also like to acknowledge rest of the JYK group: Mikko, Gusse, Paula, Teppo, Riky, Laura, Tiina, Mikael, Erik, Irene, Alexi, and Ingo. I would also like to express my gratitude to our lovely porter Meeri Salmela. Great thanks belongs to my former colleagues in Kumpula; Jari, Erika and Taru. I learned a lot with you guys! Especially I want to thank Jari for giving me the courage to take the leap to Medicinal Chemistry.

I am grateful to all my friends for giving balance into my life. My volleyball mates; you make my dark winter evenings bearable for me. I want to thank Hape for always believing in me, and taking care of me and our kids. Finally, Luka and Veini, you have really made me to take my mind off work, thanks for being my children!

Helsinki, October 2015

Raisa Haavikko

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## List of original publications

This thesis is based on the following publications:

- I R. Haavikko, A. Nasereddin, N. Sacerdoti-Sierra, D. Kopelyanskiy, S. Alakurtti, M. Tikka, C. L. Jaffe, J. Yli-Kauhaluoma; Heterocycle-fused Lupane Triterpenoids Inhibit *Leishmania donovani* Amastigotes, *Med. Chem. Commun.*, **2014**, 5, 445–451.
- II T. Parkkari, R. Haavikko, T. Laitinen, D. Navia-Paldanius, R. Ryttilähti, M. Vaara, M. Lehtonen, S. Alakurtti, J. Yli-Kauhaluoma, T. Nevalainen, J. R. Savinainen, J. T. Laitinen; Discovery of triterpenoids as reversible inhibitors of  $\alpha/\beta$ hydrolase domain 12 (ABHD12), *PLoS One*, **2014**, 9(5): e98286.
- III V. Härmä, R. Haavikko, J. Virtanen, I. Ahonen, H.-P. Schukov, S. Alakurtti, E. Purev, H. Rischer, J. Yli-Kauhaluoma, V. M. Moreira, M. Nees, K.-M. Oksman-Caldentey; Optimization of Invasion-specific Effects of Betulin Derivatives on Prostate Cancer Cells through Lead Development, *PLoS One*, **2015**, 10(5): e0126111.
- IV M. Laavola, R. Haavikko, M. Hämäläinen, T. Leppänen, R. Nieminen, S. Alakurtti, J. Yli-Kauhaluoma, V. M. Moreira, E. Moilanen; Betulin derivatives effectively suppress inflammation *in vitro* and *in vivo*, submitted.

The publications are referred to in the text by their Roman numerals.

## Author's contributions

- I. RH carried out the major part of the design, synthesis and characterization of the compounds. Structure-activity relationships were analyzed by RH. The manuscript was written by RH with contributions from co-authors.
- II. RH and SA carried out the design, synthesis and characterization of the compounds. The manuscript was written by TP, RH, and JTL with contributions from co-authors. TP and RH contributed equally.
- III. RH and SA carried out the design, synthesis and characterization of the compounds. Structure-activity relationships were analyzed by RH. The manuscript was written by VH, RH, and MN with contributions from co-authors. VH and RH contributed equally.
- IV. RH carried out the major part of the design, synthesis and characterization of the compounds. Structure-activity relationships were analyzed by RH. The manuscript was written by ML and RH with contributions from co-authors. ML and RH contributed equally.

## Abbreviations

2-AG	2-arachidonoylglycerol
2D	two dimensional
3D	three dimensional
ABHD12	serine hydrolase $\alpha/\beta$ -hydrolase domain containing 12 gene
ABHD6	serine hydrolase $\alpha/\beta$ -hydrolase domain containing 6 gene
Ac	acetyl
Bcl-2	B-cell lymphoma 2
Bn	benzyl
Bz	benzoyl
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
COX-2	cyclooxygenase-2
DCM	dichloromethane
DIPEA	<i>N,N</i> -diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FAAH	fatty acid amide hydrolase
hABHD12	human serine hydrolase $\alpha/\beta$ -hydrolase domain containing 12 gene
HIV	human immunodeficiency virus
HNE	human neutrophil elastase
IBX	2-iodoxybenzoic acid
IFN- $\gamma$	interferon- $\gamma$
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
LPS	lysophosphatidylserine
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
mPGES-1	microsomal prostaglandin E2 synthase-1
mRNA	messenger ribonucleic acid
NF- $\kappa$ B	nuclear factor kappa B
NSAID	nonsteroidal anti-inflammatory drug
NO	nitric oxide
PCC	pyridinium chlorochromate
PGE <sub>2</sub>	prostaglandin E2
Ph	phenyl
PHARC	polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, cataract –disorder
<i>p</i> -TsOH	<i>para</i> -toluenesulfonic acid
rt	room temperature
SAG	sodium antimony gluconate

SAR	structure-activity relationship
<i>t</i> -Bu	<i>tert</i> -butyl
TFAA	trifluoroacetic acid anhydride
THF	tetrahydrofuran
THL	tetrahydrolipstatin
THP	tetrahydropyranyl
TNF- $\alpha$	tumor necrosis factor $\alpha$
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate

Table 1. The cell lines mentioned in this work.

Type	Cell line
<b>Glia</b>	C6 (rat)
<b>Kidney</b>	HEK-293 (human)
<b>Leukemia</b>	CEM (human)
<b>Lung</b>	A549 (human)
<b>Macrophages</b>	THP-1 (human), J774, RAW264.7 (murine)
<b>Melanoma</b>	MEL-1, MEL-2, MEL-4 (human)
<b>Prostate</b>	Ep156T, LNCaP, PC-3 (human)

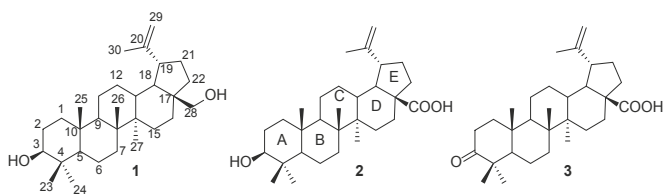


# 1 Introduction

Natural products have been used as medicines for thousands of years, and they still play an important role in modern drug discovery.<sup>1,2</sup> During a thirty-year period, from 1981 to 2010, 64% of small-molecule approved drugs were natural products, their semisynthetic derivatives or synthetic natural product mimics and analogs.

Triterpenes are secondary metabolites found all over the plant kingdom.<sup>3</sup> They are synthesized in plants by the cyclization of squalene, which contains six isoprene units. Over 20,000 triterpenes, occurring in their free form or as glycosides (saponins, in other words) have been isolated so far. Tetracyclic and pentacyclic triterpenes are the most abundant triterpenes. Pentacyclic triterpenes are divided into groups based on the chemical structures of their carbon skeletons; gammaceranes, hopanes, lupanes, oleananes, and ursanes, to name just a few. They can be found in higher concentrations in bark; in the waxy covering of leaves; or in peel. Triterpenoids show activity with several different biological targets.

Betulin (**1**, Figure 1) is a triterpenoid of the lupane series, and it can be isolated from the bark of white birches (*Betula* spp.) in approximately 30% yield of dry weight.<sup>4</sup> Birch bark is a low-value waste product of the forestry industry in the Northern Hemisphere. Many [bio]synthetic oxidation products of betulin such as betulinic acid (**2**) and betulonic acid (**3**) have several pharmacological properties. They have shown antiviral<sup>5</sup> (including anti-HIV<sup>6</sup>), anti-inflammatory<sup>7</sup>, anti-malarial<sup>8</sup>, anti-leishmanial<sup>9</sup>, and anti-tumoral<sup>10</sup> effects, among others. Betulin (**1**) can easily be modified at the C-3 secondary hydroxy group, C-20 double bond, and C-28 primary hydroxy group. These functionalities can be utilized to synthesize more active and specific betulin derivatives. In the following review of the literature only selected triterpenoid derivatives against cancer, inflammation, and leishmaniasis are covered.



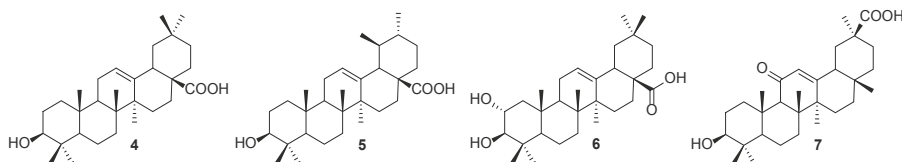
**Figure 1** Structures of betulin **1**, betulinic acid **2**, and betulonic acid **3**.

## 2 Review of the literature

### 2.1 Triterpenoids against leishmaniasis

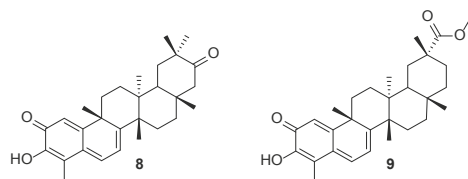
Leishmaniasis is a tropical disease caused by the protozoan *Leishmania* parasites, which are transmitted by the bite of infected sandflies.<sup>11</sup> There are over twenty *Leishmania* species causing leishmaniasis. The main forms of the disease are visceral, cutaneous, and mucocutaneous leishmaniasis. The visceral form is fatal if not treated; the cutaneous form causes skin lesions leading to scars and disability; and the mucocutaneous form destroys the mucous membrane of the nose, mouth, and throat. There are 310 million people living in areas at risk of infection. It has been estimated that there are 1.3 million new cases and 30 000 deaths annually caused by leishmaniasis. The current treatments have severe adverse effects, require hospitalization, or are highly expensive. Resistance is also a problem in some areas. One treatment does not work for all forms of the disease and species of leishmaniasis. Encouragingly, some new compounds have been shown to be selectively active against some *Leishmania* species.

Anti-leishmanial activity of betulinic aldehyde *in vitro* was first reported against *Leishmania amazonensis*.<sup>12</sup> Oleanolic acid (**4**) and ursolic acid (**5**, Figure 2) showed *in vitro* activity against *L. donovani*, *L. major*, and *L. amazonensis* promastigotes and amastigotes, i.e. extra- and intracellular forms<sup>13,14</sup> However, they showed cytotoxicity towards non-parasitized macrophage-like RAW 264.7 cells and mouse peritoneal macrophages as well, which limits their use as anti-leishmanial compounds. In another study, oleanolic acid (**4**) and maslinic acid (**6**) showed activity against *L. infantum* and *L. amazonensis* promastigotes *in vitro*.<sup>15</sup> Triterpenoids with a hydroxy group at C-2 were as active against *L. amazonensis* promastigotes as the parent oleanolic (**4**) and ursolic (**5**) acids.<sup>14</sup> On the other hand, they showed no activity against amastigotes. Oleanolic acid (**4**) was more active *in vitro* against *L. donovani* promastigotes than the corresponding C-3 methyl ether.<sup>16</sup> BALB/c mice infected with *L. donovani* were cured of their parasite burden with 18 $\beta$ -glycyrrhetinic acid (**7**).<sup>17</sup> *L. donovani* infection in BALB/c mice differs from the infection in humans, being just chronic rather than fatal, and decrease in the parasite burden is part of the course of the infection. Nevertheless, these authors consider that this is a good experimental model for the disease. Combination therapy with sodium antimony gluconate (SAG) and glycyrrhizinic acid inhibited SAG-resistant *Leishmania* infection in a synergistic manner.<sup>18</sup>



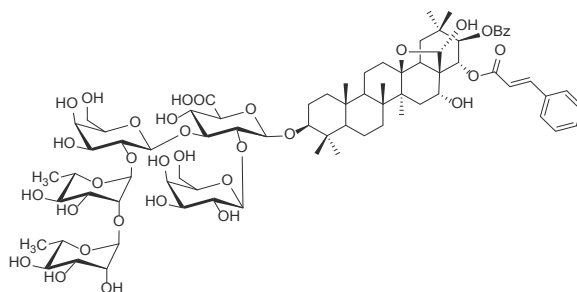
**Figure 2** Structures of oleanolic acid (**4**), ursolic acid (**5**), maslinic acid (**6**) and 18 $\beta$ -glycyrrhetinic acid (**7**).

Maytenin (**8**) and pristimerin (**9**, Figure 3) showed potent activity against *L. amazonensis* and *L. chagasi* promastigotes *in vitro* (0.09 nM and 0.05 nM for *L. amazonensis*, and 0.46 nM and 0.41 nM for *L. chagasi*, respectively).<sup>19</sup> These compounds are very potent, since the positive control pentamidine showed IC<sub>50</sub> values of 6.8 nM for *L. amazonensis*, and 4.0 nM for *L. chagasi*.



**Figure 3** Structures of maytenin **8** and pristimerin **9**.

Triterpenoid saponins with an oleanane skeleton extracted from *Maesa balansae* Mez<sup>20</sup>, and with a hederin skeleton isolated from ivy (*Hedera helix* L.)<sup>21</sup>, showed nanomolar anti-leishmanial activity *in vitro* on *L. infantum*. Oleanane saponins were also effective against *L. donovani* *in vitro*.<sup>22</sup> Both the ester and sugar moiety seemed to be important for activity (Figure 4).<sup>23</sup> In hamsters, the oleanane saponin showed similar potency to liposomal amphotericin B, which is used as a first line treatment for leishmaniasis.<sup>24</sup> Also, the triterpenoid saponin, arborenin, isolated from an Indian tree *Careya arborea* Roxb. showed *in vitro* activity against *L. donovani*.<sup>25</sup>



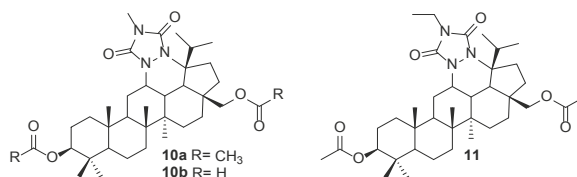
**Figure 4** Structure of an oleanane saponin extracted from *Maesa balansae* Mez with IC<sub>50</sub> value of 0.013 nM against *Leishmania infantum* amastigotes *in vitro*.<sup>23</sup>

The bark of cork oak (*Quercus suber* L.) is an abundant source of cork for industry.<sup>26</sup> As a by-product of making corkboard, the so-called black wax is obtained with no current practical or economic use. Friedelane triterpenes were extracted from it and their derivatives were synthesized. Some of them showed activity against *L. infantum*.

Dihydrobetulinic acid has been shown to cause apoptosis in *Leishmania donovani* promastigotes and amastigotes by inhibiting DNA topoisomerases.<sup>27</sup> Adding different ester groups at the C-28 position of betulinic acid (**2**)<sup>28</sup>, betulin (**1**) or dihydrobetulin<sup>29</sup>

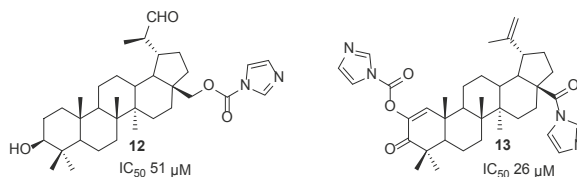


improved the anti-leishmanial activity. The presence of a carbonyl group at C-3 and a carboxyl group at C-28 seemed important for activity.<sup>28</sup> Esterification or oxidation of C-3 hydroxy group of betulinic acid increased potency against *L. amazonensis*, whereas these modifications did not make any difference in potency against *L. braziliensis*.<sup>30</sup> On the other hand, betulinic acid methyl ester showed improved activity against *L. braziliensis* compared to betulinic acid (**2**). Heterocycloadducts of betulin (**10a** and **11**) showed better inhibition than betulin (**1**) with *L. infantum* and *L. donovani* (Figure 5).<sup>9,31</sup> Compound **10b** showed the best inhibition with *L. braziliensis*.<sup>32</sup>



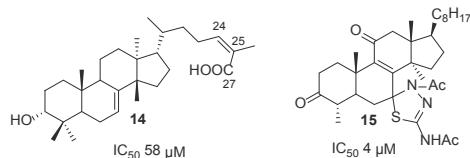
**Figure 5** The most active heterocycloadduct compounds against *L. donovani* (**10a**, IC<sub>50</sub> 9  $\mu$ M), *L. braziliensis* (**10b**, IC<sub>50</sub> 0.3  $\mu$ M) and *L. infantum* (**11**).<sup>9,31</sup>

Modification at C-20 appeared to improve anti-leishmanial activity of betulin derivatives (Figure 6).<sup>33</sup> However, imidazole derivative of betulonic acid (**13**) showed better activity. Combining betulin or betulinic acid derivative treatment with approved leishmaniasis medication, miltefosine had a synergistic effect, as IC<sub>50</sub> values were smaller than for the compounds alone. Combination treatment could be a new solution for combating the developing drug resistance.



**Figure 6** The most active betulin (**12**) and betulonic acid (**13**) derivatives against *L. infantum*.<sup>33</sup>

Based on activity studies of a series of tirucallane triterpenoids, it was concluded that the C-3 hydroxy group and a double bond at C-24/C-25 with the carboxyl group at C-27 are crucial features for antileishmanial activity against *L. infantum* (Figure 7).<sup>34</sup> The natural product tirucallane (**14**) was more effective than its semisynthetic derivatives. Among a set of tetracyclic triterpenoids, compounds with ketone or hydroxy groups at C-3, C-7, and C-11 showed higher activity against *L. infantum* promastigotes *in vitro* (Figure 7, compound **15**).<sup>35</sup>



**Figure 7** The structures of the representative tirucallane (**14**), and friedelane derivative (**15**) with their  $IC_{50}$  values against *L. infantum* promastigotes.<sup>34,35</sup>

In summary, hydrogen bond forming groups such as carbonyl or hydroxy in triterpenoid structure appear to be beneficial for anti-leishmanial activity. Many natural products are more active than their semisynthetic derivatives. Surprisingly, further development of some highly active natural products have not been reported. In the absence of an extensive SAR study, including bioisosteres of seemingly important groups, further conclusions about SARs are hard to draw. Since there are so many forms of the disease and species of *Leishmania* parasites, in addition to the parasite's complex life cycle, leishmaniasis is a highly challenging target to tackle and more research on it is needed.

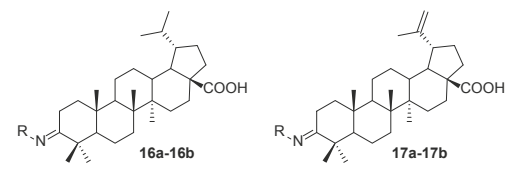
## 2.2 Pentacyclic triterpenoids against cancer

The anti-tumoral and cytotoxic properties of betulin (**1**) and betulinic acid (**2**) were already known some forty years ago.<sup>36–38</sup> Extensive studies started after finding that betulinic acid (**2**) induces apoptosis in human melanoma cells (MEL-2) without toxicity.<sup>10</sup> Initially, betulinic acid (**2**) was thought to be selectively cytotoxic only towards melanoma cells. However, when a different set of cancer cell lines was used, betulinic acid derivatives also displayed cytotoxicity towards them.<sup>39</sup> Thus far several cancer lines have been shown to be sensitive not only to betulinic acid (**2**), but also to other pentacyclic triterpenoids.<sup>40</sup> They show cytotoxicity with non-cancerous cells as well, but those tolerate much higher triterpenoid concentrations than cancer cells from the same origin.<sup>41</sup> Triterpenoids induce apoptosis via several different mechanisms, as extensively described in literature.<sup>40,42–44</sup> For example, betulinic acid (**2**) induces apoptosis by direct mitochondrial perturbations; by modulating expression levels of Bcl-2 family proteins; and by inducing NF- $\kappa$ B (nuclear factor kappa B) activation, to mention just a few of them.

Now we turn to the effects of the modifications at different positions of the pentacyclic triterpene skeleton. Since dozens of different cancer cell lines have been assayed in many studies, only the generally most promising activities are reviewed here without detailed lists of the cell lines used. It should also be noted that due the variation of experimental conditions from study to study, results are not entirely comparable to each other.

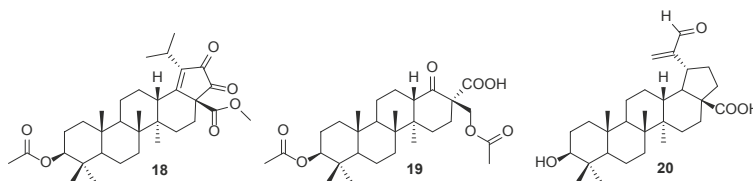
The double bond at C-20 was shown not to be important for cytotoxic activity (Figure 8).<sup>45,46</sup> In fact, when the double bond at C-20 was reduced, activity was further improved.<sup>39</sup> Modifying the C-20 double bond into a ketone, hydroxy or oxime group resulted in loss of activity.<sup>39</sup> In another study, compounds having the reduced C-20 double bond in the presence of C-3 oxime (**16a**) or phenylhydrazone (**16b**) moieties showed

increased activity against some of the cell lines tested, compared to the corresponding derivatives possessing the double bond (**17a** and **17b**, respectively, Figure 8).<sup>45</sup> A phenylhydrazone or oxime group at C-3, and a 20,29-dihydro moiety seemed to be important for cytotoxicity towards prostate and ovarian, or lung cancer cell lines.<sup>47</sup> In a set of ring E-modified oxidation products cytotoxic activity was related to the presence of the  $\beta$ -dicarbonyl group (**18** and **19**) or an  $\alpha$ -unsaturated ketone/aldehyde moiety (**20**) in their structures (Figure 9).<sup>48</sup>



Compound	R	ED <sub>50</sub> (μM)
<b>16a</b>	OH	0.7 - 3
<b>16b</b>	NH-Ph	0.4 - 6
<b>17a</b>	OH	2 - 10
<b>17b</b>	NH-Ph	2 - 10

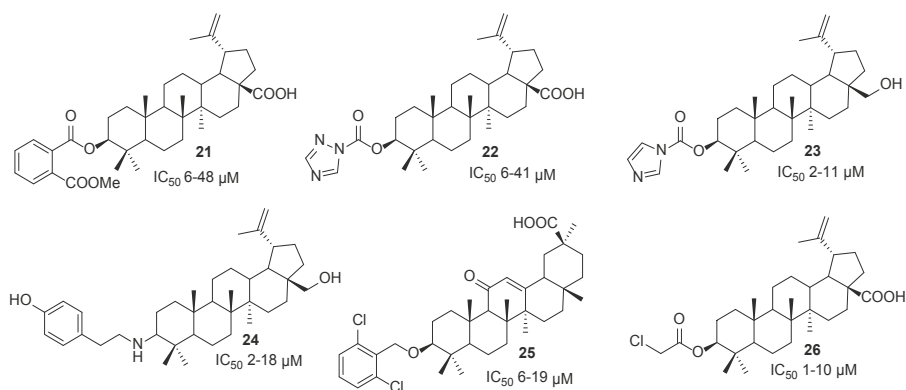
**Figure 8** Comparison of cytotoxicity of the C-19 isopropenyl and isopropyl derivatives of betulonic acid against prostate, lung, and ovarian cancer cell lines.<sup>45</sup>



**Figure 9** Representative ring E-oxidized betulin derivatives possessing a  $\beta$ -dicarbonyl group (**18** and **19**) or an  $\alpha$ -unsaturated ketone/aldehyde (**20**) moiety.<sup>48</sup>

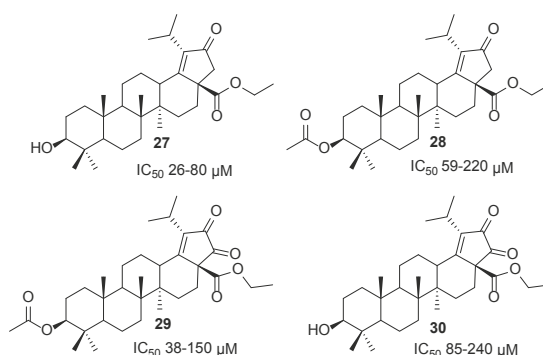
Converting the C-3 hydroxy group of betulonic acid (**2**) into a ketone increased the cytotoxicity towards several cancer cell lines.<sup>39,45,47,49,50</sup> On the other hand, betulonic acid (**3**) was less active than betulonic acid (**2**) on some other cell lines.<sup>45</sup> In general, the C-3 hydroxy group does not seem to be essential for cytotoxicity.<sup>46</sup> Derivatives of the pentacyclic triterpenoid ursolic acid with a  $3\beta$ -orientated hydroxy group were more cytotoxic than the corresponding  $\alpha$ -diastereomer.<sup>51</sup> Adding a  $3\beta$ -amino group improved cytotoxicity further. Derivatizing the C-3 hydroxy group of betulonic acid (**2**) with a phthaloyl<sup>52</sup>, (1*H*-1,2,4-triazolocarboxyl)oxy<sup>28</sup> or 1*H*-imidazole-1-carboxylate<sup>53,54</sup> group led to higher cytotoxic activity compared to betulonic acid (**2**) (Figure 10; compounds **21** - **23**, respectively). The 3-aza derivatives of betulin (**24**) and betulonic acid showed improved cytotoxicity compared to betulonic acid (**2**).<sup>55</sup>

The structurally-related glycyrrhetic acid derivatives possessing a benzyl ether substituent at C-3 (**25**) showed improved cytotoxicity compared to glycyrrhetic acid, and caused cell death via apoptosis.<sup>56</sup> Betulinic acid derivatives having C-3 ethyl carbamates showed higher cytotoxicity than phenyl carbamates.<sup>57</sup> Compounds possessing an alkyl ester group at C-3 showed only moderate activity, whereas an ester with a chloroacetyl side chain (**26**) was a more potent cytotoxic compound than the alkyl ester substituted ones.<sup>58</sup> Interestingly, betulin derivatives with glyceryl esters linked to C-3 showed lower cytotoxic activity than betulin (**1**).<sup>59</sup>



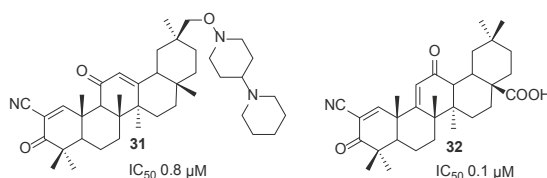
**Figure 10** Representative compounds with different variations at C-3 and their  $IC_{50}$  values from cytotoxicity studies towards leukemia, melanoma, colon cancer and prostate cancer cell lines.<sup>52-56,58</sup>

Betulinic acid<sup>60</sup> and ursolic acid<sup>51,61,62</sup> derivatives possessing a 3-*O*-acetyl moiety had improved cytotoxic activity compared to the parent compounds. On the other hand, 3 $\beta$ -hydroxy-21-ketone (**27**) was more active than 3 $\beta$ -acetoxy-21-ketone (**28**), whereas 3 $\beta$ -acetoxy-21,22-dione (**29**) was more active than its deacetylated counterpart (**30**, Figure 11).<sup>63</sup>



**Figure 11** *3β-Hydroxy-21-ketone (27), 3β-acetoxy-21-ketone (28), 3β-acetoxy-21,22-dione (29) and 3β-hydroxy-21,22-dione (30) and their  $IC_{50}$  values towards leukemia, melanoma, colon and prostate cancer cell lines.<sup>63</sup>*

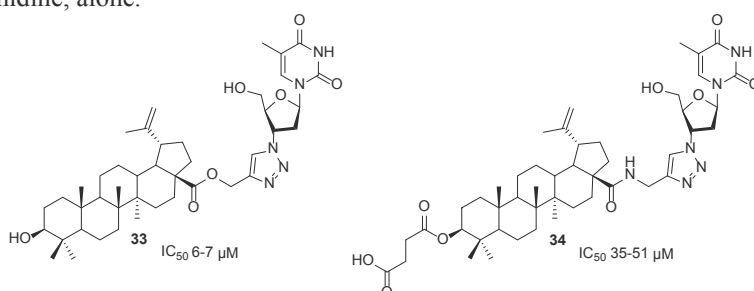
Ring A-fused heterocyclic betulin and allobetulin derivatives showed decreased cytotoxicity, and the authors presume it is due to their poor aqueous solubility and resulting bioavailability.<sup>64</sup> However, ring A-fused pyrazine derivative showed cytotoxic activity against several cancer cell lines.<sup>65</sup> Interestingly, when a pyrazine ring was fused to ring E or five-membered ring A, the resulting compounds were inactive. Adding a cyano-enone moiety to ring A of the 18β-glycyrrhetic acid derivative (**31**) improved antiproliferative activity against leukemia cells compared to the parent compound (Figure 12).<sup>66</sup> Also, oleanolic acid derivatives with a cyano-enone-bearing ring A (**32**) have shown potency against lung cancer<sup>67</sup> and leukemia<sup>68</sup>. The methyl ester of compound **32** induces apoptosis of cancer cells and has been tested in Phase II clinical trials.<sup>69,70</sup> Compounds with a lupane skeleton possessing an electron-withdrawing group at C-2 showed improved activity against human melanoma.<sup>71</sup> Lupane derivatives with a C-2 methylene group and a C-28 carbonyl showed good cytotoxicity towards several cancer cell lines.<sup>72</sup>



**Figure 12** *Structures of the 18β-glycyrrhetic acid derivative **31** and oleanolic acid derivative **32**, and their  $IC_{50}$  values towards a myelocytic leukemia cell line.<sup>66,68</sup>*

It has been shown in several studies that a protonated carboxyl group at C-28 is important for cytotoxic activity against several cancer cell lines.<sup>45,46,49,53,71,73–75</sup> In some studies, it has been concluded that a hydrogen bond donor is important at C-28 for cytotoxic activity.<sup>49,51</sup> However, others have shown that it is the C-28 carbonyl group that is necessary for cytotoxicity.<sup>46,51,75,76</sup> When azidothymidine was linked via a triazole ring to

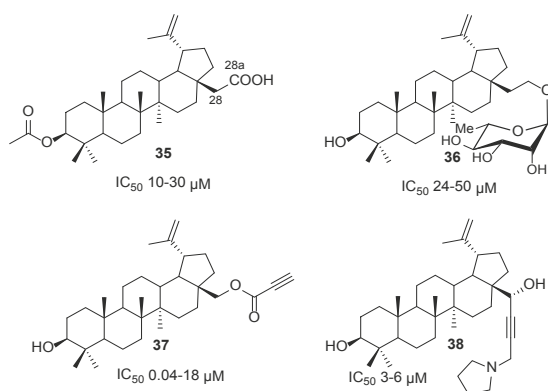
a C-28 ester (**33**) or amide (**34**), triterpenoids showed good to moderate cytotoxic activity, respectively (Figure 13).<sup>77,78</sup> It is noteworthy that these hybrid compounds displayed more potent cytotoxic activity than either of the parent compounds, betulinic acid (**2**) or azidothymidine, alone.



**Figure 13** Representative azidothymidine derivatives of betulinic acid and their  $IC_{50}$  values towards cervical cancer cell lines.<sup>77,78</sup>

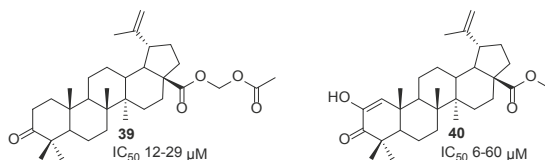
C-28 glycidic amide and  $\beta$ -ketoesters were more potent than betulinic acid (**2**).<sup>60</sup> Results were in line with the assumption that a C-28 carbonyl group is important for the activity. Betulonic aldehyde derivatives with triazole or pyrazole moieties attached to the C-28 carbonyl showed selective cytotoxicity towards colon cancer and breast cancer cells.<sup>79</sup> Betulinic acid saponin bearing L-rhamnose sugars at C-3 and C-28 showed higher cytotoxicity (1.3-1.9  $\mu$ M) compared to betulinic acid (**2**).<sup>80</sup> On the other hand, compounds having a glyceryl unit at C-28 were as potent as betulinic acid (**2**).<sup>59</sup>

Compound **35** (Figure 14), with an elongated C-17 side chain, was more active than the corresponding compound with a C-3 hydroxyl, namely ichopanic acid.<sup>81</sup> Ichopanic acid was more active than betulinic acid (**2**). Other compounds with elongated C-17 side chains were cytotoxic only when a primary hydroxy group was present at C-28a. Surprisingly, coupling L-rhamnose to C-28a (**36**) resulted in decreased cytotoxicity. Compounds possessing a short alkynyl chain linked to C-28 with ester (**37**) or carbonate groups showed strong cytotoxicity against human leukemia.<sup>82</sup> In addition, compounds with an alkynyl side chain with a pyrrolidine moiety as a small terminal tertiary amino moiety directly attached to C-28 (**38**) showed increased cytotoxicity compared to betulinic acid (**2**).<sup>83</sup>



**Figure 14** Representative C-28 modified betulin derivatives and their  $IC_{50}$  values towards leukemia, ovarian, breast, and colon cancer cell lines.<sup>81–83</sup>

An ethynyl side chain at C-28 showed very promising antitumor activity by triggering apoptosis.<sup>79</sup> Betulin derivatives with an acetylenic side chain at C-28 showed improved cytotoxicity compared to betulinic acid (**2**).<sup>74</sup> In a set of different C-28 ester derivatives of betulinic acid, the acetoxymethyl ester derivative (**39**) showed similar cytotoxic activity to the parent acid whereas other esters were inactive (Figure 15).<sup>84</sup> Ester derivatives with a vicinal dicarbonyl system conjugated in its enol form on ring A (**40**) were the most active compounds. It was concluded that modifications of ring A are more efficient than ester modifications at C-28.



**Figure 15** Representative acetoxymethyl ester (**39**) and dicarbonyl (conjugated enol form) (**40**) derivatives of betulinic acid with their  $IC_{50}$  values from cytotoxicity studies towards leukemia, prostate, lung, and breast cancer cell lines.<sup>84</sup>

Triterpenoids have obvious and general water solubility problems that are likely to lead to a low bioavailability of the compounds. To improve the aqueous solubility and bioavailability of betulin derivatives, some of them have been encapsulated with soybean lecithin, resulting in increased cytotoxicity.<sup>72,74,79</sup> Betulinic acid derivatives with an amino acid conjugated to C-28 showed selective cytotoxicity on melanoma cells and remarkably improved aqueous solubility.<sup>85</sup> In a set of ursolic acid derivatives, more lipophilic compounds showed higher cytotoxicity, which might be due to their better membrane permeability.<sup>62</sup>

To summarize: the pentacyclic triterpene skeleton can be modified at many different positions in order to obtain more cytotoxic compounds towards various cancer cell lines.

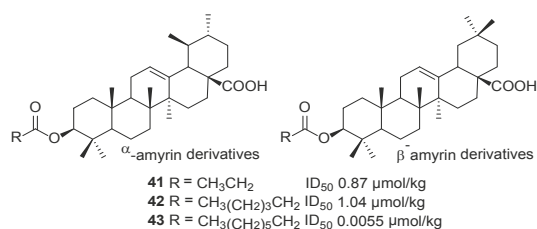
Cytotoxic activity of betulin-derived triterpenoids is typically more related to the electronic and hydrogen bond-forming properties than to the size of the substituents. In many studies, the sets of the compounds tested were quite small, so further conclusions concerning structure-activity relationships are challenging to draw based on those results. To be certain about the importance of the presence of a particular group, corresponding bioisosteres should be synthesized and tested as well. In mechanistic studies, several mechanisms of action have been found.<sup>86,87</sup> One compound can act via several mechanisms, which makes further development into a potential lead compound harder.

## 2.3 Triterpenoids as analgesics and anti-inflammatory agents

The function of inflammation is to repair damage or an infection, and thus return the organism back to homeostasis.<sup>88</sup> However, if inflammation prolongs, it can cause damage to the organism. Chronic inflammation is known to lead to cancer development. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to delay the development of premalignant tumors in some instances.<sup>89</sup> The inflammation cascade is very complex and involves many different classes of mediators.<sup>88</sup> Inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) are some of the enzymes responsible for the inflammatory response. Excessive expression of iNOS or COX-2 have been associated with many diseases, such as neurodegenerative diseases<sup>90,91</sup> and rheumatoid arthritis<sup>92</sup>, as well as carcinogenesis<sup>93</sup>. Natural triterpenoids have been shown to inhibit inflammation<sup>94-96</sup> and later on they were shown to hinder the expression of pro-inflammatory mediators, the production of secondary messengers, and expression of transcription factors.<sup>88</sup>

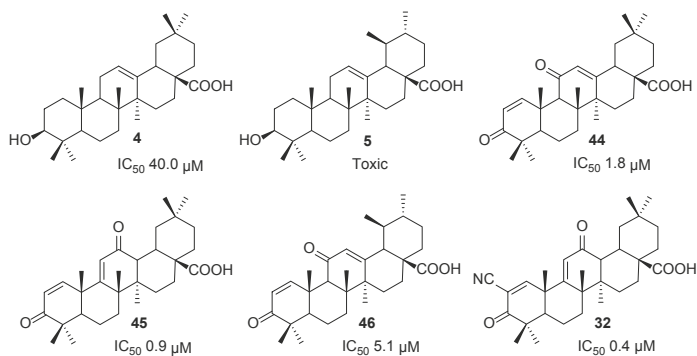
Betulinic acid (**2**) has shown inhibition of carrageenan and serotonin-induced rat paw edema, which demonstrates anti-inflammatory properties.<sup>7</sup> Betulin (**1**) has also been shown to reduce acute LPS (lysophosphatidylserine)-induced lung injury.<sup>97</sup> Based on a set of natural triterpenoids, it was concluded that a carboxyl group at C-28 or C-30 increases the inhibition of carrageenan paw and TPA (12-*O*-tetradecanoylphorbol-13-acetate) ear edema.<sup>98</sup> When mixtures of  $\alpha$ - and  $\beta$ -amyrin ester derivatives were tested on an acetic acid-induced visceral pain model, propionate (**41**), hexanoate (**42**), and octanoate (**43**) derivatives showed very good antinociceptive activity, compound **43** being the best of the tested compounds (Figure 16).<sup>99</sup>





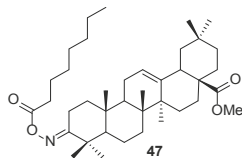
**Figure 16** The most active  $\alpha$ - and  $\beta$ -amyrin derivatives against acetic acid-induced visceral pain.<sup>99</sup>

In a set of oleanolic (4) and ursolic acid (5) derivatives (Figure 17), the ring A enones were found to be crucial for inhibition of iNOS and COX-2 mRNA (messenger ribonucleic acid) expression.<sup>100,101</sup> The presence of a carbonyl group at C-11 or C-12 of ring C seemed important for the activity. Addition of an  $\alpha$ -double bond (44 and 45) enhanced inhibitory activity further (Figure 17). Compounds with a hydrophilic group at C-28 were more active than compounds with hydrophobic groups. Compounds with an oleanane skeleton were found to show higher inhibition than compounds with an ursane skeleton (46). In further studies, a C-2 nitrile group (compound 32) was found to further improve the inhibition of NO production induced by IFN- $\gamma$  (interferon- $\gamma$ ) in mouse macrophages.<sup>102</sup> A methyl ester of 32 has been shown to suppress inflammatory signals in cancer and immune cells.<sup>103</sup> It has been concluded that electron-withdrawing groups at C-2 increase potency, whereas electron-releasing groups decrease potency.<sup>102</sup> According to a structure-activity study of hundreds of synthetic oleanane triterpenoids, the ring A enone unit is critical for maximum inhibition of iNOS induction.<sup>104</sup> Expansion, opening, and heterocycle installation at ring A decreases activity. The presence of ester and amide groups at C-28 results in excellent activity; and a cyano group at C-28 leads to picomolar inhibitory activity of induced iNOS expression.



**Figure 17** Structures of oleanolic and ursolic acid (4 and 5), and their representative derivatives (44-46 and 32) with their IC<sub>50</sub> values for inhibition of IFN- $\gamma$ -induced NO production in mouse macrophages.<sup>100,102</sup>

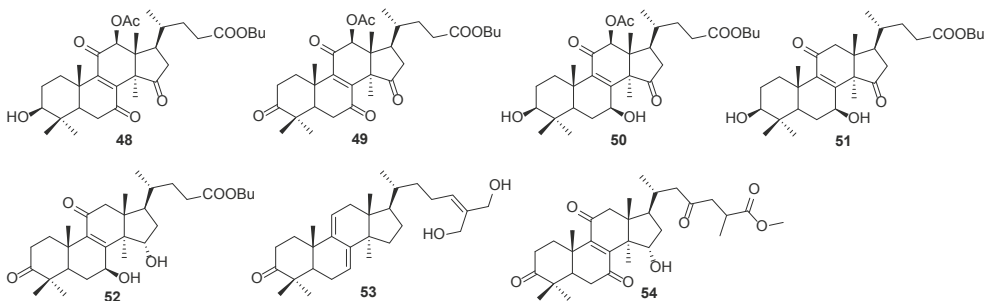
Methyl 3-octanoyloxyiminoolean-12-en-28-oate (**47**) showed analgesic activity at high doses (300 mg/kg) and an anti-inflammatory effect comparable to the effect of diclofenac on carrageenan-induced edema (Figure 18).<sup>105</sup> Compound **47** showed a synergistic analgesic effect with morphine and a synergistic anti-inflammatory effect with diclofenac.



**Figure 18** Structure of methyl 3-octanoyloxyiminoolean-12-en-28-oate (**47**).<sup>105</sup>

Pentacyclic triterpenoids, oleanolic acid (**4**), ursolic acid (**5**), glycyrrhetic acid (**7**), glycyrrhizin, betulinic acid (**2**) and lupeol were shown to inhibit human neutrophil elastase (HNE), which assists neutrophil migration to inflammation sites.<sup>106</sup> Ursolic acid (**5**) showed the most potent inhibitory activity with an  $IC_{50}$  value of 5.5  $\mu$ M. Interestingly, the tetracyclic triterpenoids tested showed only minor inhibition. Based on the *in vitro* and docking studies, a C-28 carboxyl group and double bond at C-12 increased the inhibitory activity.

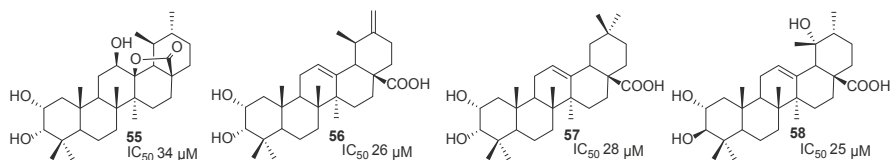
Triterpenoids present in apple peel, such as betulinic acid (**2**), oleanolic acid (**4**), ursolic acid (**5**), corosolic acid and maslinic acid (**6**) have shown anti-inflammatory activity.<sup>107</sup> Lanostane triterpenoids **48-54** showed anti-inflammatory activity by inducing heme oxygenase protein expression, which is associated with anti-inflammatory responses in RAW264.7 cells (Figure 19).<sup>108</sup>



**Figure 19** Lanostane triterpenoids with anti-inflammatory activity.<sup>108</sup>

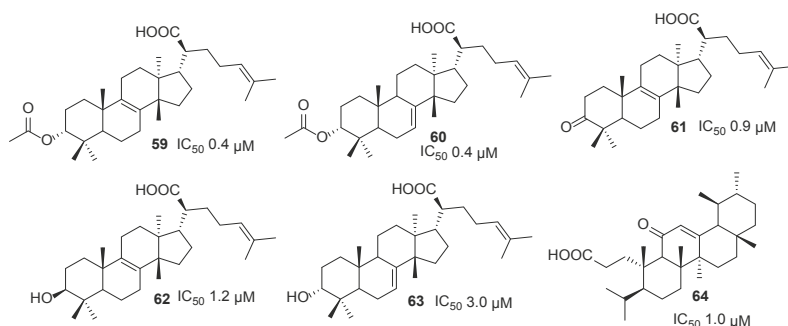
Ursolic acid (**5**) and methyl ursolate showed significant reduction of carrageenan-induced paw edema.<sup>109</sup> Betulinic acid-peptide conjugates containing histidine, alanine, tryptophan and isoleucine amino acid fragments showed anti-inflammatory activity comparable to that of indomethacin.<sup>110</sup> Pristimerin (**9**) has been shown to inhibit LPS-induced  $TNF-\alpha$  and

IL-8 production.<sup>111</sup> Oleanane and ursane-type natural compounds **55–58** showed moderate inhibition of LPS-induced NO production (Figure 20).<sup>112</sup>



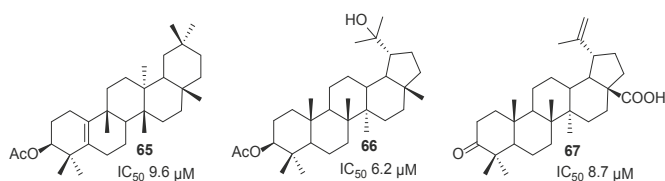
**Figure 20** The oleanane and ursane type natural compounds with their  $IC_{50}$  values against LPS-induced NO production.<sup>112</sup>

A set of triterpene carboxylic acids were tested as inhibitors of microsomal prostaglandin  $E_2$  synthase (mPGES-1), which is an inducible pro-inflammatory enzyme.<sup>113</sup> Tirucallic acids showed the best  $IC_{50}$  values (Figure 21). Acetylation of the C-3 hydroxy group improved activity (compounds **59** and **60**). Neither converting the C-3 hydroxy group into ketone (compound **61**) nor changing  $3\alpha$ - configuration of the hydroxy group to  $3\beta$ - (compound **62**) improved the activity. Among the roburic acids, a ketone (**64**) showed mPGES-1 inhibition while roburic acids devoid of it showed no activity ( $IC_{50} > 10 \mu$ M).



**Figure 21** Representative tirucallic acids (**59–63**) and a roburic acid derivative (**64**) with their  $IC_{50}$  values for inhibition of mPGES-1 activity.<sup>113</sup>

The bark, leaves and flowers of a Thai plant use in herbal medicine, *Hiptage benghalensis* (L.) Kurz, have traditionally been used to treat wounds, ulcers and asthma among other ailments. Triterpenes **65–67** (Figure 22) isolated from this plant were shown to suppress NO and  $PGE_2$  production, and iNOS and COX-2 expression in LPS-induced RAW 264.7 macrophages through NF- $\kappa$ B suppression.<sup>114</sup>

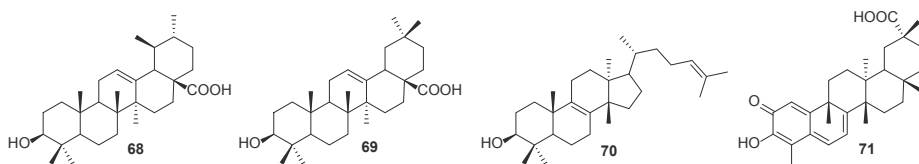


**Figure 22** Triterpenes isolated from *Hiptage benghalensis* and their  $IC_{50}$  values against LPS-induced NO production in RAW 264.7 macrophages.<sup>114</sup>

### *Triterpenes showing activity against cannabinoid receptor mediated pain and inflammation*

Two types of cannabinoid receptors have been identified so far, namely cannabinoid 1 (CB1) and cannabinoid 2 (CB2).<sup>115</sup> The CB1 receptors are distributed in the peripheral and central nervous system, and CB2 receptors are mainly found in immune and hematopoietic system. Many cannabinoid receptor agonists have been shown to reduce inflammation and neuropathic pain.<sup>116</sup> The first endogenous ligands, or endocannabinoids, discovered were anandamide and 2-arachidonoylglycerol (2-AG).<sup>115</sup> 2-AG is metabolized by serine hydrolase enzymes monoacylglycerol lipase (MAGL) and  $\alpha/\beta$ -hydrolases 6 (ABHD6) and 12 (ABHD12). Signaling system of these endocannabinoids has been shown to participate in many dysfunctions, such as pain, inflammation, immunological disorders, obesity and cancer.<sup>117</sup>

Betulinic acid (**2**) has been shown to bind to CB1 and CB2 receptors; it can inhibit breast cancer growth via this pathway.<sup>118</sup>  $\alpha$ - and  $\beta$ -amyrin (Figure 23, **68** and **69** respectively) have been shown to reduce chronic and inflammatory pain directly via CB1 and CB2 receptors.<sup>116</sup> Later on,  $\alpha$ - and  $\beta$ -amyrin were found to selectively modulate CB1 receptor activation and down-regulate endocannabinoid hydrolases.<sup>119</sup> Anti-inflammatory and anti-neuropathic hyperalgesia effects of euphol (**70**) depend partly on its interactions with cannabinoid receptors.<sup>120</sup> Interestingly, despite these antinociceptive effects, euphol (**70**) does not show any psychomodulatory effects in the central nervous system, even at high doses. Pristimerin (**9**), euphol (**70**), and  $\beta$ -amyrin (**69**) have been reported to suppress 2-AG hydrolysis by inhibiting MAGL activity ( $IC_{50}$  = 93 nM, 315 nM, and 2.8  $\mu$ M respectively).<sup>121,122</sup> Celastrol (**71**) has been shown to cause analgesia through CB2 signaling.



**Figure 23** Structures of  $\alpha$ -amyrin **68**,  $\beta$ -amyrin **69**, euphol **70**, and celastrol **71**.

In summary: inflammation is a truly complex process and several different mediators are involved, which makes discovery of new anti-inflammatory compounds very challenging. Assays generally involve extensive animal testing or *in vitro* experiments on several separate enzymes. Up to now, mainly natural triterpenes have been tested, and typically the assayed sets of compounds have included very few derivatives. Since the assays and targets vary greatly, and the number of the tested compounds is low, it is nearly impossible to find any meaningful SAR related to anti-inflammatory effects of triterpenoids. Only oleanolic acid derivatives have been studied extensively enough to conduct SAR data as addressed above.<sup>104</sup> However, some individual compounds show enough inhibition of inflammation mediators to be good targets for further development.

### 3 Aims of the study

The aim of the study was to modify functional groups of the betulin skeleton to improve the biological activities of the starting natural compounds betulin (**1**), betulinic acid (**2**) and betulonic acid (**3**). In addition, the aim was to find betulin-derived compounds with high potency and good selectivity for further development.

The more specific aims of the study were

- to design and synthesize heterocycle-fused betulin derivatives which are more active against *Leishmania donovani* (I)
- to design and synthesize inhibitors of the serine hydrolase  $\alpha/\beta$ -hydrolase 12 (ABHD12) and to generate a pharmacophore model for ABHD12 inhibitors based on lupane-type triterpenoids (II)
- to design and synthesize anti-invasive betulin derivatives against prostate cancer cell models (III)
- to design and synthesize betulin derivatives with anti-inflammatory effects and study their effects *in vitro* and *in vivo* (IV)

## 5 Results and discussion

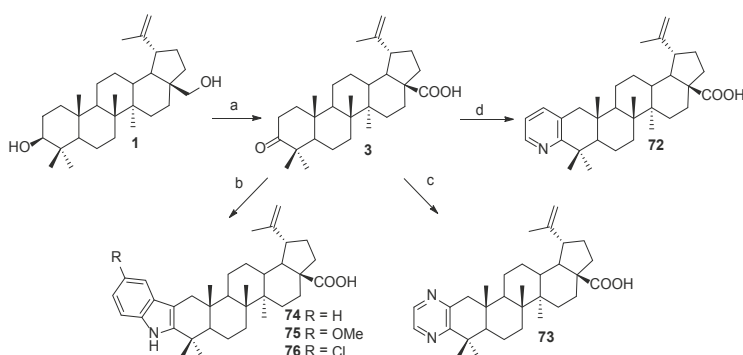
### 5.1 Anti-leishmanial activity of betulin derivatives (I)

#### 5.1.1 Synthesis of betulin derivatives for activity studies against *Leishmania donovani*

Previously our group had found that heterocyclic betulin derivatives possess promising anti-leishmanial activity on *L. donovani*, which causes the most severe form of leishmaniasis.<sup>9,28</sup> Based on those results, we wanted to further explore effects of different kinds of fused heterocyclic betulin derivatives with varying groups at C-28 on *L. donovani* axenic amastigotes. Axenic amastigote assay has proven to be a quick, reliable and affordable screening method.<sup>123</sup>

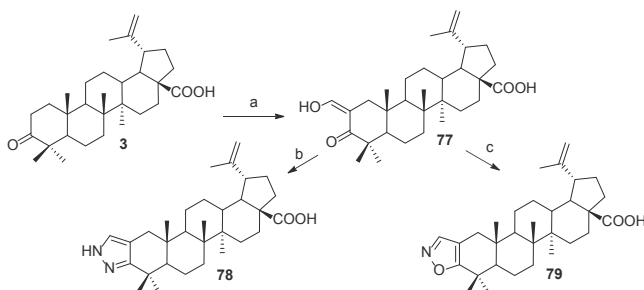
It should be noted that none of the synthesis routes were optimized, because our objective was to produce sufficient amounts of the target compounds for biological testing. A detailed presentation of the materials, synthetic, and analytical methods can be found in the original Publication I and in the associated supporting information.

The natural product betulin (**1**) was used as a starting point for all syntheses. Betulin (**1**) was oxidized by Jones oxidation to betulonic acid (**3**), which was used as a versatile synthesis intermediate (Figure 24). Even though Jones oxidation is described generally as an easy way to convert primary alcohols to carboxylic acids in one step, starting from betulin (**1**), extracting and purifying the resulting betulonic acid (**3**) was really laborious process and the isolated yields remained low. A great amount of research has been done to find better yielding, less toxic, environmentally friendlier, cheaper, and more facile methods for oxidizing betulin (**1**) into betulonic acid (**3**).<sup>124–126</sup> Nevertheless, Jones oxidation remains as the most reliable method to prepare betulonic acid (**3**) from betulin (**1**). The ketone carbonyl at C-3 of betulonic acid (**3**) was utilized in syntheses of A-ring fused heterocycles. Pyridine (**72**), pyrazine (**73**) and indole (**74-76**) derivatives were obtained from betulonic acid (**3**) in one step (Figure 24).



**Figure 24** *Synthesis of indole and pyrazine derivatives of betulonic acid. Reagents and conditions: (a)  $\text{Na}_2\text{Cr}_2\text{O}_7$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{H}_2\text{O}$ , acetone, rt, 21 h, 44%; (b) appropriate phenylhydrazine hydrochloride, acetic acid, reflux, 3 h, 21-42%; (c) 1,2-ethylenediamine, sulphur, morpholine, reflux, 21 h, 68%; (d) propargylamine,  $\text{Cu(I)Cl}$ , ethanol, reflux, 17 h, 11%.*

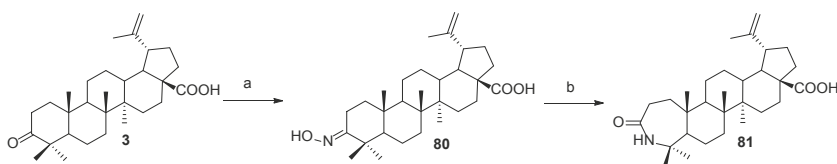
Next, betulonic acid (**3**) was converted into another useful synthesis intermediate, 2-hydroxymethylene adduct (**77**), by the Claisen condensation with ethyl formate (Figure 25). Refluxing compound **77** in acidic conditions with hydrazine hydrate gave the pyrazole derivative **78**, and its analogous reaction with hydroxylamine hydrochloride yielded the isoxazole derivative **79**.



**Figure 25** *Synthesis of pyrazole and isoxazole derivatives of betulonic acid. Reagents and conditions: (a) ethyl formate,  $\text{NaH}$ , THF, rt, 16 h, 56%; (b)  $\text{H}_2\text{NNH}_2 \cdot \text{H}_2\text{O}$ ,  $p\text{-TsOH}$ , toluene,  $150^\circ\text{C}$ , 17 h, 80%; (c)  $\text{H}_2\text{NOH} \cdot \text{HCl}$ , acetic acid, reflux, 3 h, 68%.*

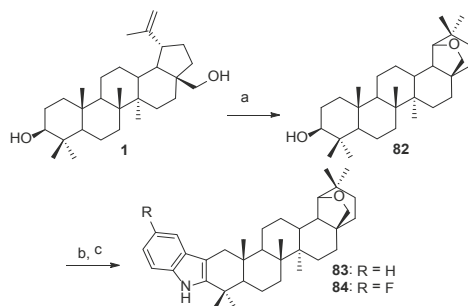
A reaction between betulonic acid (**3**) and hydroxylamine hydrochloride produced an oxime derivative **80**, which was converted in the presence of trifluoroacetic anhydride (TFAA) to the target 7-membered A-ring lactam (**81**) in the classical Beckmann rearrangement (Figure 26).





**Figure 26** Synthesis of lactam derivative of betulonic acid. Reagents and conditions: (a)  $H_2NOH \cdot HCl$ , pyridine, methanol, reflux, 16 h, 84%; (b) TFAA, DCM (dichloromethane), rt, 20 h, 33%.

Refluxing betulin (**1**) in formic acid rearranges it into allobetulin (**82**; Figure 27).<sup>127</sup> The oxidation of allobetulin into allobetulone works well by Jones oxidation. Indole derivatives of allobetulin (**83**, **84**) were synthesized by the Fischer indole synthesis in the same fashion as described above for using betulonic acid (**3**) as a starting material. The compounds derived from allobetulin (**82**) and allobetulone are less soluble than the corresponding betulonic acid derivatives, which may complicate carrying out biological assays. This should be kept in mind when interpreting biological results, since lower activity may not result from different structural features, but the lower solubility of the test substances in the assay system. In any case, these compounds could help studying the significance of the C-28 carboxyl group.

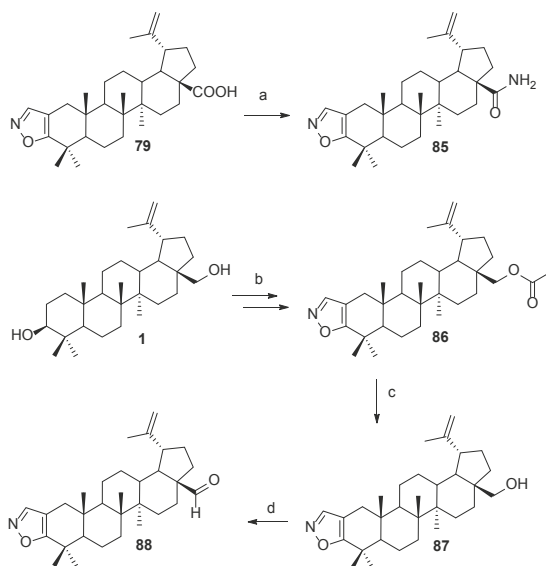


**Figure 27** Synthesis of indole derivatives of allobetulin. Reagents and conditions: (a) formic acid, reflux, 45 min; 1 M KOH in ethanol, benzene, 30 min, 25%; (b)  $Na_2Cr_2O_7$ ,  $H_2SO_4$ ,  $H_2O$ , acetone, rt, 21 h, 88%; (c) appropriate phenylhydrazine hydrochloride, acetic acid, reflux, 2 h, 19-57%.<sup>1</sup>

Based on the literature, the carboxyl group at C-28 appears to be important for most of the bioactivities betulin derivatives have been reported to possess. However, only a few different kind of variations have been made for the C-28 position of the lupane skeleton, and a related comprehensive study with bioisosteres is still missing. The reason for the lack of such a study is probably the fact that it would require laborious multistep syntheses when, in general, only simple one-step syntheses are used to derivatize natural compounds. However, a tiny step towards studying effects of different groups at C-28 was taken with a set of isoxazole derivatives of betulin. We synthesized isoxazole derivatives

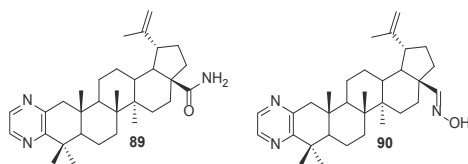
of betulin with primary amide (**85**), acetoxy (**86**), hydroxy (**87**), and aldehyde (**88**) groups at C-28 (Figure 28).

Compound **79** was first converted into the corresponding acyl chloride, which was treated with aqueous ammonia to obtain the primary amide **85**. The primary hydroxy group of betulin **1** was protected as tetrahydropyranyl (THP) ether allowing a selective oxidation of the C-3 hydroxy group by pyridinium chlorochromate (PCC). In strongly acidic conditions of the subsequent isoxazole ring-forming step, the THP group was replaced with an acetoxy group. The subsequent deprotection and oxidation reactions gave the hydroxy and aldehyde derivatives, respectively (Figure 28).



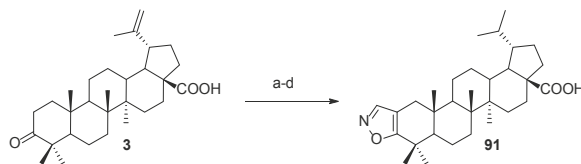
**Figure 28** C-28 modifications of the isoxazole derivative of betulonic acid. Reagents and conditions: a: Starting from **79**: (I) oxalyl chloride, DCM, rt, 1 h; (II) 25% aqueous ammonia, CHCl<sub>3</sub>, rt, 30 min, yielding **85**, quant. b: Starting from betulin (**1**):(I) pyridine *p*-toluenesulfonate, 3,4-dihydro-2H-pyran, DCM, rt, 18 h, 80%; (II) PCC, DCM, rt, overnight, 46%; (III) ethyl formate, NaH, THF, rt, 22 h, 52%; (IV) H<sub>2</sub>NOH•HCl, acetic acid, reflux, 4 h, yielding **86**, quant.; c: Continuing from **86**: *p*-TsOH, methanol, reflux, 20 h, yielding **87**, quant.; d: Continuing from **87**: IBX, THF, DMSO, rt, 3.5 h, yielding **88**, 51%.

Our modification studies of pyrazine derivatives comprised only C-28 primary amide (**89**) and oxime groups (**90**; Figure 29). Compound **90** was obtained by oxidizing betulin (**1**) to betulonic aldehyde followed by the formation of a pyrazine ring, and finally the conversion of an aldehyde group to an oxime.



**Figure 29** C-28 modifications of a pyrazine derivative of betulonic acid. Reagents and conditions for **89**: Starting from lupa-2,20(29)-dieno[2,3-*b*]pyrazin-28-oic acid (**73**): (a) oxalyl chloride, DCM, rt, 3 h; (b) aqueous ammonia, DCM, rt, 1 h, quant.; for **90**: Starting from betulin (**1**): (a) PCC, DCM, rt, 1 h, 27%; (b) 1,2-ethylenediamine, sulfur, morpholine, reflux, 2.5 h, 17%; (c).  $H_2NOH \cdot HCl$ , pyridine/ethanol (1:3), reflux, 16 h, 77%.

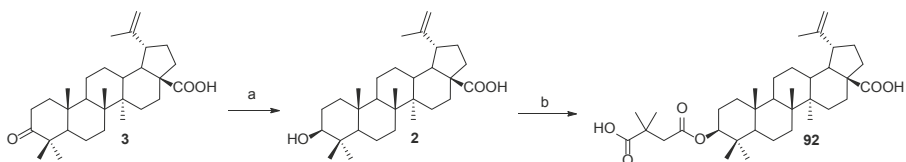
In some studies, the corresponding compounds with an isopropenyl or isopropyl group at C-19 gave different activity results. To study whether the isoxazole derivatives of betulonic acid follow a similar pattern, compound **91** was synthesized (Figure 30). Initially we had serious troubles in our attempts to reduce the C-20 carbon-carbon double bond of betulonic acid (**3**). Reduction of the double bond worked fine with betulon (**1**) (in methanol/ THF 2:1) but, due to low yield of the following Jones oxidation, this route was not feasible. Next, reduction of the isopropenyl group was carried out for betulonic acid methyl ester and its carbon-carbon double bond was reduced successfully; but here we hit the wall with cleavage of the ester. When we used benzyl ester as the protecting group, we got a 1:1 mixture of starting material and hydrogenated product. The solvent used was changed from THF to ethyl acetate, resulting in the single desired product. Later, a similar reduction was carried out for betulonic acid (**3**) in ethyl acetate, and even after prolonged reaction time, little bit of starting material was still present. Hence, using benzyl protection was the most practical route to obtain an isopropyl moiety at C-19. The subsequent isoxazole synthesis worked equally well as before.



**Figure 30** Synthesis of isoxazole derivative of dihydrobetulonic acid. Reagents and conditions: (a) benzyl bromide,  $K_2CO_3$ , DMF, 55 °C, 22 h, 43%; (b)  $H_2$ , 10% Pd/C, ethyl acetate, rt, 72 h, quant.; (c) ethyl formate, NaH, THF, rt, 22 h, 56%; (d)  $H_2NOH \cdot HCl$ , acetic acid, reflux, 6 h, 90%.

*Leishmania*-human immunodeficiency virus (HIV) co-infections have been reported from 35 endemic countries. This co-infection causes more severe forms of leishmaniasis which are more difficult to treat, therefore those people infected have very high mortality rates.<sup>11</sup> Keeping this in mind, we synthesized a known anti-HIV agent bevirimat, 3 $\beta$ -(3-carboxy-3-methylbutanoyloxy)lup-20(29)-en-28-oic acid (**92**) to explore its effects on *L. donovani*

(Figure 31). Several different conditions, including microwave irradiation, different temperatures, solvents and catalysts were tried and eventually a method was found to produce a sufficient amount of compound **92** for biological tests.

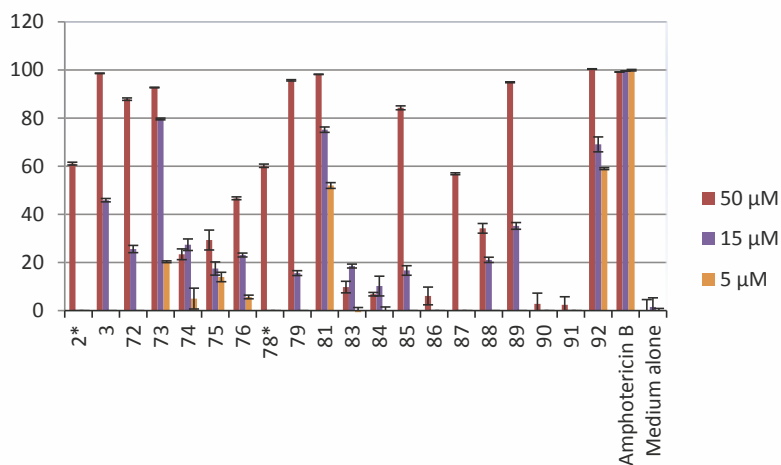


**Figure 31** Synthesis of bevirimat. Reagents and conditions: (a)  $\text{NaBH}_4$ , 2-propanol, rt; (b) 2,2-dimethylsuccinic anhydride, DIPEA (*N,N*-diisopropylethylamine), DMF 170 °C, 2 d, 5%.

### 5.1.2 Biological profiling of the experimental set of betulin derivatives against *Leishmania donovani*

Leishmanicidal activity of the compounds was assayed by alamarBlue viability assay on axenic amastigotes. Assays were started at 50  $\mu\text{M}$  concentration following with an assay at 15  $\mu\text{M}$  for compounds with more than 70% inhibition at 50  $\mu\text{M}$ . The most potent derivatives were assayed at 5  $\mu\text{M}$  concentration.

Among the isoxazole derivatives (**78**, **85-88**, and **91**) inhibition at higher 50  $\mu\text{M}$  concentration varied a lot (Figure 32). However, all the compounds having higher activity at 50  $\mu\text{M}$  showed similar inhibition to each other at lower 15  $\mu\text{M}$  concentration. All of them had lower activity than betulonic acid (**3**).



**Figure 32** Average inhibition activity of triplicates of the tested compounds on axenic amastigotes of *Leishmania donovani*. Amphotericin B was tested at 1  $\mu\text{M}$  as a positive control. \*Compounds **2** and **78** precipitated at 50  $\mu\text{M}$ .<sup>†</sup>

Pyrazine derivatives **73** and **89** showed good inhibition even at lower concentrations. Interestingly, an exception to this trend was the pyrazine derivative with a C-28 oxime group (**90**); it had almost no activity at all, in contrast to the previous studies, where oxime derivatives showed good inhibition at 50  $\mu\text{M}$ .<sup>28</sup>

Among the tested compounds, three of them, **73**, **80**, and **92**, were found to have significant inhibition of parasite growth at 5  $\mu\text{M}$  concentrations. On the other hand, compounds also showed slight THP-1 macrophage toxicity.

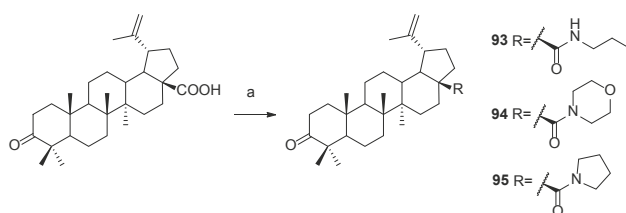
When comparing these results to our previous ones, some improvement can be seen.  $\text{IC}_{50}$  values of the three most active compounds in this study were 4 - 13  $\mu\text{M}$ , whereas in our earlier study these values varied in the range of 9 - 30  $\mu\text{M}$ .<sup>9</sup> However, in order to obtain more potent betulin derivatives against *L. donovani*, further optimization and improvement are clearly needed.

## 5.2 Betulin derivatives as serine hydrolase (ABHD)12 inhibitors (II)

ABHD12 is one of the enzymes hydrolyzing 2-AG, which is a natural ligand for cannabinoid receptors, i.e. an endocannabinoid.<sup>128</sup> Mutations in the ABHD12 gene cause PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, cataract) disorder, which is a neurodegenerative disease leading to cataract, hearing loss, and ataxia, to name few symptoms.<sup>129</sup> Since the crystal structure of ABHD12 is not known, developing new ligands is challenging. Abundant triterpenes serve as a good starting point for the discovery of new ABHD12 inhibitors, as they have shown several related activities. Previously certain triterpene derivatives were found to inhibit human ABHD12 hydrolase activity reversibly. For example, pristimerin (**9**) has been shown to inhibit MAGL activity *in vitro*<sup>121,130</sup>, and  $\alpha$ -amyrin (**68**) and  $\beta$ -amyrin (**69**) have been shown to reduce inflammatory and neuropathic hyperalgesia via cannabinoid receptors.<sup>116</sup>

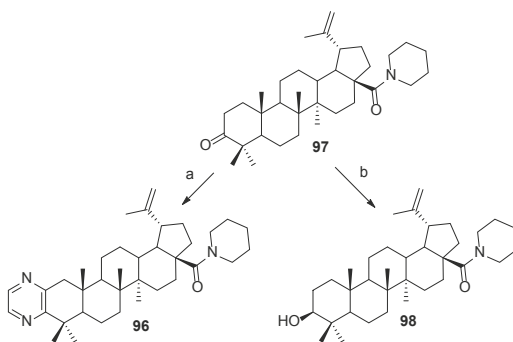
### 5.2.1 Synthesis of C-28 and ring A-modified betulin derivatives

Previously our group had synthesized different kinds of ester and ether derivatives of betulinic acid.<sup>5</sup> In addition to those compounds and derivatives for the *Leishmania* studies described above, the carboxyl group of betulonic acid (**3**) was converted further into different amides (Figure 33). A detailed presentation of the materials, synthetic, and analytical methods can be found in the original Publication II and in the associated supporting information.



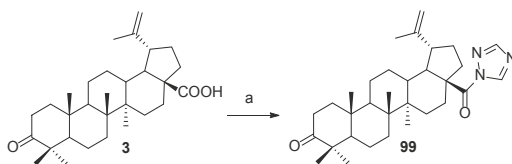
**Figure 33** *Synthesis of amide derivatives of betulonic acid. Reagents and conditions: (a) (1) DCM, oxalyl chloride, rt, 5 h. (2) DCM, appropriate amine, rt, 19 h, yield 32-47%.*

In the series of C-28-modified compounds, a pyrazine derivative with C-28 tertiary amide (**96**) was also synthesized (Figure 34). We wanted to make a compound with tertiary amino group at C-28; actually, we found that zinc acetate in the presence of triethoxysilane had been reported to *selectively* reduce tertiary amides to amines, even in the presence of a ketone.<sup>131</sup> Interestingly, and to our surprise, in the case of betulonic acid derivative **97** only the ketone was reduced. The amide stayed intact, resulting in compound **98** as a product of the reaction.



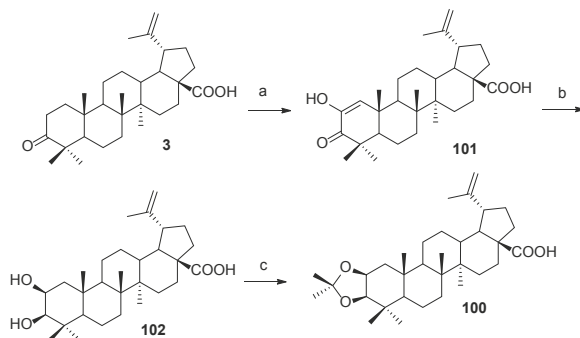
**Figure 34** *Synthesis of compounds 96 and 98. Reagents and conditions: (a) 1,2-ethylenediamine, sulphur, morpholine, reflux, 2 h, yield 67%; (b)  $\text{Zn}(\text{OAc})_2$ , triethoxysilane, THF, 40 °C, 1.5 h, yield 57%.*

Since it is known that some triazole ureas inhibit serine hydrolases<sup>132</sup>, a 1,2,4-triazole derivative of betulonic acid (**99**) was synthesized (Figure 35). To avoid a hydrolytically labile urea structure, a triazole amide was synthesized instead.



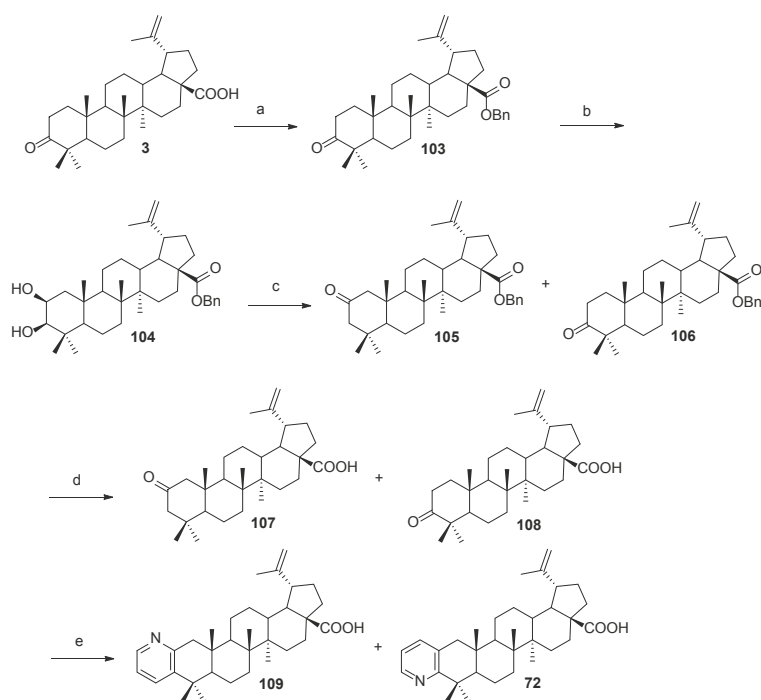
**Figure 35** Synthesis of 1-(1H-1,2,4-triazol-1-yl)betulonone **99**. Reagents and conditions: (a) 1,1'-carbonyldi(1,2,4-triazole), THF, reflux, 20 h, yield 49%.

To diversify the structures of A ring-fused heterocycles, compound **100** was synthesized. Betulonic acid **3** was first oxidized to **101** which, in turn, was reduced to the diol **102**. The formed diol was protected as an acetal to yield heterocyclic compound **100**. Its tentative metabolite **102** was also tested. Moreover, **101** proved to be a useful intermediate for other syntheses.



**Figure 36** Synthesis of compound **100**. Reagents and conditions: (a) *t*-BuOK, *t*-BuOH, 40 °C, air, 2 h, yield quant.; (b) NaBH<sub>4</sub>, ethanol, THF, 0 °C, 1.5 h, yield 74%; (c) pyridinium *p*-toluenesulfonate, 2,2-dimethoxypropane, rt, 22 h, yield 70%.

To study the effects of the position of nitrogen atoms in pyridine and pyrazine derivatives, a pyridine derivative possessing a nitrogen atom at C-2 was synthesized. First, the ketone carbonyl at position C-3 had to be moved to C-2. After protecting the carboxyl group at C-28 to yield **103**, the C-3 ketone was converted into a diol **104**. The diol **104** was converted to a tosylate at 30 °C. When the reaction temperature was increased to 60 °C, the tosylate participated in an elimination reaction that gave a mixture of two ketones (**105** and **106**). In the literature, this method is described for betulin (**1**) only whereas, for the first time, we used betulonic acid (**3**) as the starting compound.<sup>133</sup> Despite several attempts, we were unable to separate the two ketones as benzyl esters (**105** and **106**) or carboxylic acids (**107** and **108**) before making the actual pyridine derivatives (**109** and **72**).



**Figure 37** Synthesis of the pyridine-fused lupane skeleton with the nitrogen atom in position C-2. Reagents and conditions: (a) Benzyl bromide,  $K_2CO_3$ , DMF, 55 °C, overnight, 43%; (b) (i) *t*-BuOK, *t*-BuOH, 40 °C, air, 2 h, yield quant.; (ii)  $NaBH_4$ , EtOH, THF, 0 °C, 1.5 h, yield 74%; (c) *p*-toluenesulfonyl chloride, dry pyridine, 30 °C for 6 h, 60 °C for 18 h, yield 58%, mixture of compounds **105** and **106**; (d)  $H_2$ , 10% Pd/C, THF, 4.5 h, yield 80%, mixture of compounds **107** and **108**; (e) propargylamine,  $CuCl_2$ , ethanol, reflux, 17 h, yield 21% of compound **109**.


### 5.2.2 Structure-activity relationships of the tested compounds with ABHD12 hydrolase

In our preliminary studies, ursolic acid (**5**) was unexpectedly recognized as a selective serine hydrolase ABHD12 inhibitor with insignificant effect on ABHD6 and MAGL.<sup>11</sup> Encouraged by this finding, a set of triterpenoids was designed and tested. Starting with 15 commercially available triterpenoids, it was demonstrated that the triterpene skeleton and the C-28 carboxyl group are important for human ABHD12 (hABHD12) inhibition. Since oleanolic (**4**), ursolic (**5**) and betulinic acid (**2**) had only minor differences in their inhibitory activities, it was concluded that their characteristic substitution pattern and the size of ring E have no effect on hABHD12 inhibition; so betulinic acid derivatives were chosen for further studies. Structures of all the tested compounds can be found in Publication II and in the Supplemental Material of Publication II.

A series of betulinic acid derivatives was used to study further structural features needed for hABHD12 inhibition. The necessity of a carboxyl group at C-28 was



confirmed by testing C-28 aldehydes (**88** and **110**), esters (**113-115**, and **118**), ether (**112**), and amides (**93-95**, **98**), which showed only moderate to no potency (Figure 38). In addition, changing the carboxyl group to an oxime (**111**) lowered the potency ( $IC_{50}$  6.5  $\mu$ M). Furthermore, potency was lowered when the C-3 hydroxy group of betulinic acid (**2**) was replaced with an oxime group (**80**;  $IC_{50}$  12.0  $\mu$ M). Finally, compound **117** possessing oxime groups at both C-3 and C-28 positions had enhanced potency ( $IC_{50}$  4.0  $\mu$ M), but not at the same level as betulinic acid (**2**;  $IC_{50}$  2.5  $\mu$ M).



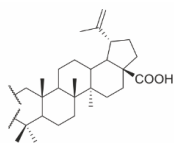
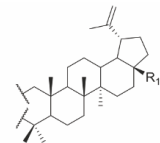













Comp.	R <sub>1</sub>	Inhibition % at 10 $\mu$ M ( $\pm$ s.e.m.)	$IC_{50}$ ( $\mu$ M)	Comp.	R <sub>2</sub>	R <sub>3</sub>	Inhibition % at 10 $\mu$ M ( $\pm$ s.e.m.)	$IC_{50}$ ( $\mu$ M)
<b>2</b>			2.5	<b>80</b>				12.0
<b>98</b>		6 ( $\pm$ 0.5)		<b>93</b>			16 ( $\pm$ 1.5)	
<b>110</b>		29 ( $\pm$ 0.8)		<b>94</b>			23 ( $\pm$ 2.0)	
<b>111</b>			6.5	<b>95</b>			20 ( $\pm$ 0.3)	
<b>112</b>		NI		<b>99</b>			23 ( $\pm$ 1.1)	
<b>113</b>		15 ( $\pm$ 0.5)		<b>116</b>				4.4
<b>114</b>		NI		<b>117</b>				4.0
<b>115</b>		NI		<b>118</b>			NI	
				<b>119</b>			NI	

**Figure 38** Structures and inhibition activities of the tested aldehyde, ester, ether, amide and oxime derivatives of betulin in hABHD12-HEK293 lysates.<sup>11</sup>

The effects of ring A modification were also studied (Figure 39). Adding a hydroxy group to C-2 showed similar inhibition to the parent compound betulinic acid (**2**). All ring A-fused heterocycles showed good inhibition. Changing the ring A into a lactam ring or adding a 2,2-dimethyldioxolane moiety showed modest inhibition. Inhibition was

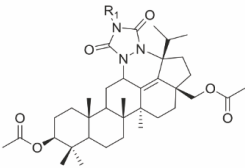
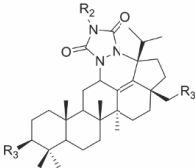























improved slightly by fusing a pyridine ring with the nitrogen atom at C-3. Inhibition was improved even further when another nitrogen atom was introduced to the fused ring at the C-2 position, *i.e.* pyrazine ring. The importance of the carboxyl group at C-28 was also seen here, as inhibition decreased significantly when it was replaced with a primary or tertiary amide moiety. The fused pyridine ring with a nitrogen atom at C-2 showed only very low inhibition. Since the ring A-fused isoxazole derivative showed low inhibition as well, it seems that a hydrogen bond donor or acceptor at the C-3 position is important for the enzymatic inhibition.

Fusing an indole or a pyrazole ring to the lupane skeleton improved inhibition, the indole derivative (**74**) being the most active compound with an  $IC_{50}$  value of 0.9  $\mu M$  (Figure 39). The inhibition potencies of indole derivatives were higher than those determined for the natural compounds maslinic acid (**6**) and oleanolic acid (**4**). Pyrazole derivative (**78**) was able to inhibit the enzyme at the same level as these natural products. The importance of the C-28 carboxyl group was observed with the indole derivative too, since inhibition was completely lost for the indole derivative of allobetulin when assayed.

									
Comp.	Ring A	IC <sub>50</sub> (μM)	Comp.	Ring A	IC <sub>50</sub> (μM)	Comp.	Ring A	R <sub>1</sub>	Inhibition % at 10 μM (± s.e.m.)
72		6.7	78		1.4	88		CHO	16 (± 1.6)
73		3.6	81		9.1	89		CONH <sub>2</sub>	31 (± 0.8)
74		0.9	100		7.5	96			9 (± 0.6)
75		1.6	102		3.7	109		COOH	34 (± 1.1)

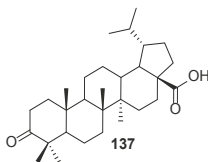
**Figure 39** Structures of the ring A-modified betulinic acid derivatives and their inhibition activities in hABHD12-HEK293 lysates.<sup>11</sup>

Fusing urazole ring with varying substituents to the lupane skeleton of betulin decreased the inhibition of serine hydrolase ABHD12 (Figure 40). Ester groups at C-28 of betulin were not tolerated either. The inhibition activities of selected urazole derivatives are presented in Figure 40.

						
Comp.	R <sub>1</sub>	Inhibition % (10 μM) (± s.e.m.)	Comp.	R <sub>2</sub>	R <sub>3</sub>	Inhibition % (10 μM) (± s.e.m.)
120		7 (± 0.2)	131	Me- 		7 (± 1.7)
121		9 (± 0.6)	132	Me- 		15 (±0.6)
122	Me- 	5 (± 0.3)	133	Me- 		5 (±0.3)
123		2 (± 1.0)	134			2 (±0.2)
124		3 (± 1.0)	135			14 (± 0.4)
125		12 (± 1.3)	136			3 (± 0.5)
126		0 (± 3.3)				
127		7 (± 0.9)				
128		18 (± 0.7)				
129		10 (± 0.4)				
130		8 (± 0.5)				

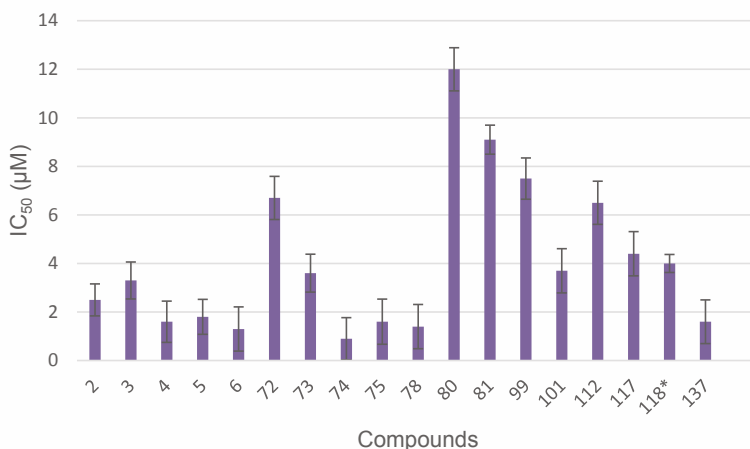
**Figure 40** Structures of urazole derivatives assayed and inhibition activities in hABHD12-HEK293 lysates.<sup>11</sup>

Dihydrobetulonic acid (**137**, Figure 41) showed improved hABHD12-HEK293 lysate inhibition (1.6 μM) compared to betulonic acid (**3**; 3.3 μM). It is noteworthy that no other targets among the metabolic serine hydrolases were found for compound **137**.



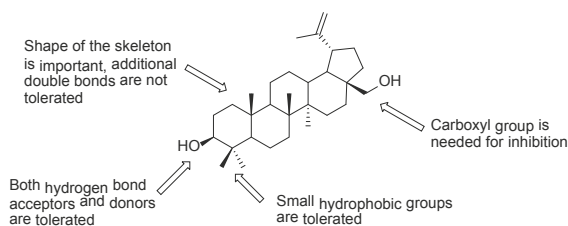
**Figure 41** The structure of the dihydrobetulonic acid **137**.

The IC<sub>50</sub> values of the most active compounds are presented in Figure 42. Compound **74** was the most active. It showed improved inhibition compared to any of the natural products tested. Compounds **75** and **78** were almost as potent and showed the same level of inhibition as the natural compounds.



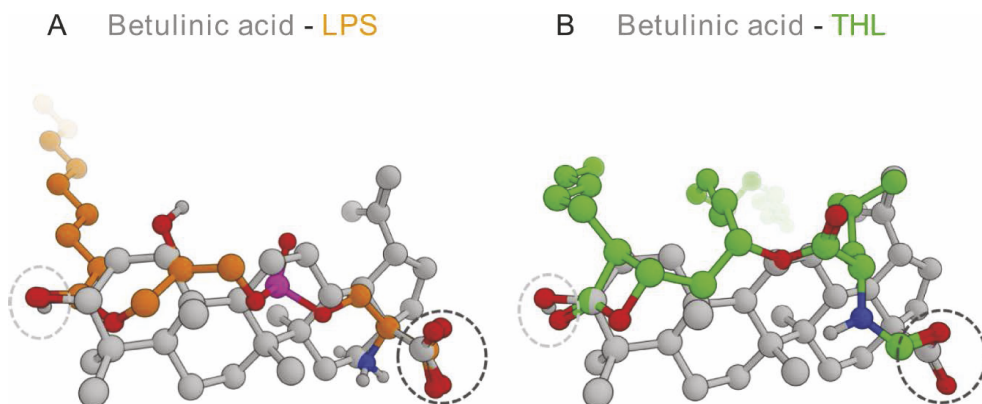
**Figure 42** IC<sub>50</sub> values of the most active triterpenoids tested in hABHD12-HEK293 lysates. Data are mean of three independent experiments. \*n=2

Important features for inhibitory activity against serine hydrolase ABHD12 are summarized in Figure 43. The shape of a triterpene skeleton plays an important role as additional double bonds, which change the shape of the molecular skeleton, resulted in no inhibition (compound **32** and celastrol **71**). The carboxyl group at C-28 is important, as all its modifications lead to lower inhibition. A small hydrophobic substituent is needed at C-4, since asiatic acid and hederagenin showed no inhibition. A hydrogen bond donor or acceptor is required at C-3 for high inhibitory activity. This was shown with pyridine and pyrazine as well as with pyrazole and isoxazole derivatives of betulinic acid.



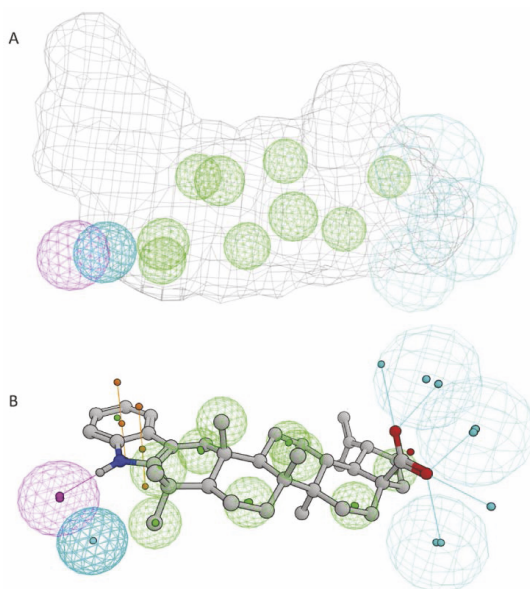
**Figure 43** Features of betulin derivatives affecting inhibition of hABHD12.

The core structure of the triterpenoid skeleton can be recognized by many proteins and therefore triterpenoids have shown activity with many targets. However, the triterpenoids assayed in this study displayed no activity on other established targets of the endocannabinoid system, such as ABHD6, MAGL, fatty acid amide hydrolase (FAAH) or the cannabinoid receptors CB1 and CB2. When the structure of betulinic acid (**2**) was superimposed with those of the *in vivo* substrate LPS and irreversible inhibitor tetrahydrolipstatin (THL), striking similarities in topology, dimensions, and distance as well as orientation of the functional groups were found (Figure 44).



**Figure 44** LPS (in A orange carbons), the *in vivo* substrate of ABHD12, and THL (in B green carbons), the irreversible inhibitor of ABHD12, superimposed with betulinic acid (**2**) (grey carbons). The carboxyl group of LPS and the formyl group of THL align with the carboxyl group of betulinic acid (**2**; dark grey circle). The ester carbonyl of LPS and the lactone carbonyl of THL align with the hydroxy group of betulinic acid (**2**; light grey circle). LPS and THL were modelled in an extended conformation and the hydrocarbon chains have been partially faded. Reprinted from II.

Sixty-eight triterpenoids were tested as ABHD12 inhibitors, and 18 compounds were classified as active. Based on the activities of these compounds, a pharmacophore model was constructed with 14 distinct pharmacophoric features (Figure 45).



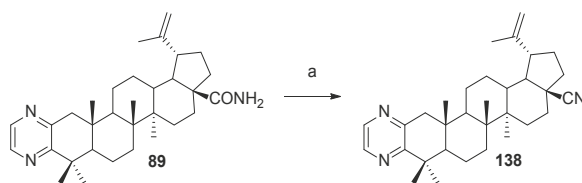
**Figure 45** *Pharmacophoric features for binding of triterpenes to ABHD12. Green: hydrophobic site, magenta: acceptor site, cyan: donor site, gray cage: ligand shape constraint. In B the best solution for the alignment of the most active compound (74) is presented. Reprinted from II.*

In conclusion, ABHD12 inhibiting natural compounds and their derivatives were reported. The best compounds inhibited hABHD12 fully with  $IC_{50}$  values in the submicromolar range. The compounds had no effect on other endocannabinoid hydrolases or cannabinoid receptors. The triterpenoids showed selective inhibition towards ABHD12. With the SAR analysis, important features for ABHD12 inhibition were determined. The first pharmacophore model was developed, based on those results.

### 5.3. Betulin derivatives active against prostate cancer cells (III)

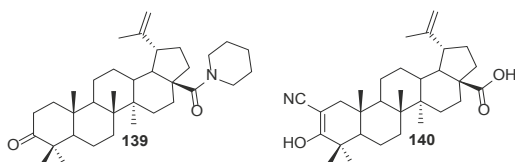
#### 5.3.1 Synthesis of betulin derivatives active against prostate cancer cells

The set of natural triterpenoids and their semisynthetic derivatives described above were tested against two prostate cancer cell lines PC-3 and LNCaP. In addition to those compounds, one more compound was synthesized to include a nitrile substituent at the C-28 position. A detailed presentation of the materials, synthetic, and analytical methods can be found in the original Publication III and in the associated supporting information. Starting from the ring A-fused pyrazine derivative of betulonic acid **73**, the C-28 carboxyl group was converted into a nitrile group via a primary amide (Figure 46).



**Figure 46** *Synthesis of compound 138. Reagents and conditions: (a) cyanuric chloride, DMF, 0 °C, 1 h, yield 84%.*

Compound **139** (Figure 47) was synthesized in same manner as described earlier for compounds **93-95**. Compound **140** was obtained from the isoxazole derivative **79** by cleaving the isoxazole ring with KOH in methanol.



**Figure 47** *Structures of the compounds 139 and 140.*

### 5.3.2 Biological profiling of the betulin derivatives on prostate cancer cells

Betulinic acid (**2**) has been identified as an inducer of apoptosis in melanoma cells (cell lines MEL-1, MEL-2 and MEL-4).<sup>10</sup> In addition, betulin (**1**) has been found to block invasion in brain and lung cancer cells (C6, and A549, respectively).<sup>134</sup> Here antineoplastic and anti-invasive properties of these compounds and their synthetic derivatives on prostate cancer cell lines (PC-3 and LNCaP) were studied. Starting with high-throughput screening, cancer-specific compounds were identified and a set of related compounds was synthesized. Modifications were made to the A ring and C-28, and these new compounds were also used in ABHD12 studies (*vide supra*).

Biological assays started with high-throughput screening using 2D (2-dimensional) cell cultures. The cell lines used were EP156T (non-transformed prostate epithelium), LNCaP (androgen sensitive prostate cancer) and PC-3 (castration-resistant invasive prostate cancer). The subsequent studies continued with 3D (3-dimensional) setting for specific anti-invasive properties. To interpret the complex image data obtained, statistical analyses were performed. Using measures of area, complexity and area of dead cells from PC-3 cultures, a dendrogram with hierarchical clustering was generated (Figure 48). The dendrogram has two main branches: one cluster includes the most potent anti-proliferative and anti-invasive compounds (in blue); the other contains weak (in green) and non-effective (in grey) compounds.



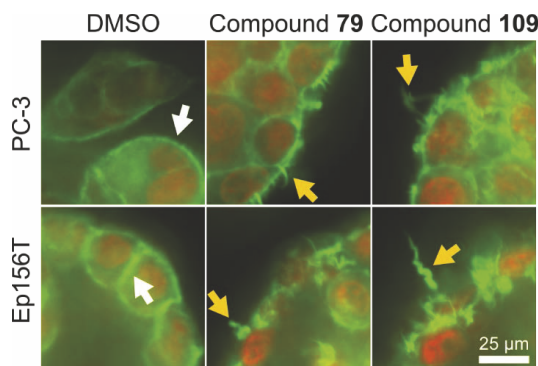


with betulonic acid derivatives: when the C-28 carboxyl group was replaced with a secondary (**93**) or tertiary amide (**94**, **95**, and **139**), activity was lost.

When the isoxazole ring of **79** was changed to a pyrazole, the resulting compound **78** was even more potent and specifically anti-invasive. The pyrazine derivative of betulonic acid (**73**) had a strong anti-invasive effect without any growth inhibition. Surprisingly, a primary amide moiety at C-28 (**89**) did not decrease the anti-invasive effect level. On the other hand, pyrazine derivatives bearing tertiary amide or nitrile groups at C-28 showed no inhibition. Interestingly, pyridine derivatives having nitrogen at C-2 or C-3 were anti-invasive at significantly lower concentrations than the pyrazine derivative. This might be due to their higher lipophilicity compared to pyrazine derivative.

In order to find out which were the most potent cytotoxic compounds instead of being anti-invasive, inhibition of proliferation and induction of cell death was studied. In PC-3 cells, most compounds did not show noticeable effects on proliferation at low concentrations (<10  $\mu\text{M}$ ). In LNCaP cells, growth inhibition and cytotoxicity started at lower concentration (3  $\mu\text{M}$ ); and in Ep156T cells, cytotoxicity was observed at even lower concentrations (0.3 – 1  $\mu\text{M}$ ). The compounds did not induce cell death specifically in PC-3 or LNCaP cells at lower concentrations than 30  $\mu\text{M}$ .

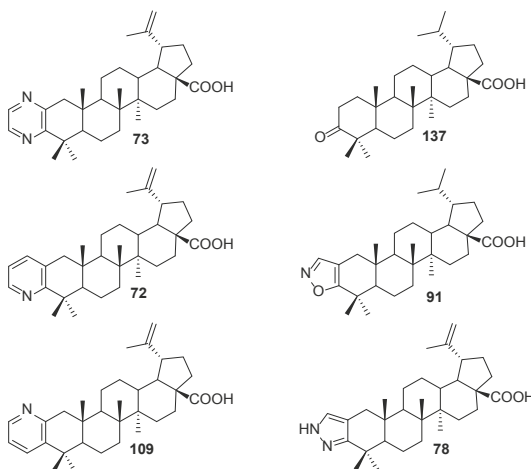
Additionally, it was confirmed by kinase phosphorylation profiling that compounds **79** and **109** do not primarily affect cell cycle progression or mitosis. They are cytotoxic only at high concentrations. At low (nanomolar) concentrations they promote anti-invasive effects. These compounds disrupt actin cytoskeleton organization, which could be observed from the corkscrew-like phenotype of the filamentous actin (Figure 49). This may be linked to efficient suppression of the invasive properties of PC-3 cells.



**Figure 49** *Confocal microscope-captured 3D morphologies of prostate spheroids exposed to compounds **79** and **109**. The actin cytoskeleton is stained green, nuclei with red. Reprinted from III.*

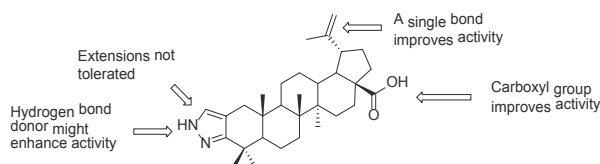
Chemical structures of the compounds showing the most potent anti-invasive effects on PC-3 cells are presented in Figure 50. These compounds did not show noticeable effects with other cell lines. Even though most of them showed growth inhibition in all cell lines

at higher ( $> 1 \mu\text{M}$ ) concentrations, they may act as specific inhibitors of invasion and motility at lower concentrations.



**Figure 50** Structures of the compounds most active against prostate cancer cell line PC-3.<sup>III</sup>

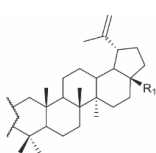
The important features for anti-invasive effects are summarized in Figure 51. Based on these results, the carboxyl group at C-28 is important for activity. A single bond at C-19 and a hydrogen bond donor at C-2' of the A ring-fused heterocycle seem to improve activity considerably. Extensions of the A ring-fused heterocycle were not tolerated.

















**Figure 51** Features needed for anti-invasive effects on the PC-3 prostate cancer cell line compared to compound 78.

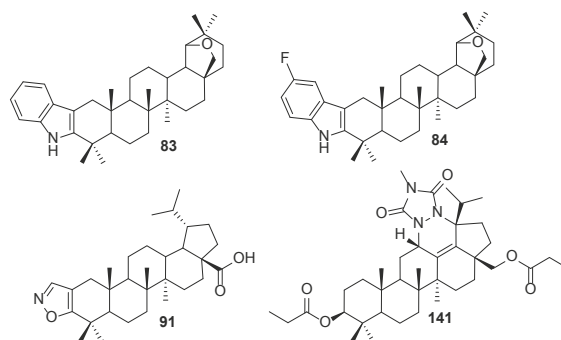
## 5.4 Anti-inflammatory effects of betulin derivatives (IV)

Encouraged by the earlier studies showing betulin (**1**) and betulinic acid (**2**) to possess anti-inflammatory properties<sup>97,7</sup> we wanted to explore the effects of heterocyclic betulin derivatives on inflammation as well. The mechanism of action behind the effects was also studied. Chemical structures of the compounds tested are given in Figures 52 and 53.



Compound	Ring A	R <sub>1</sub>	Compound	Ring A	R <sub>1</sub>
3		COOH	88		
72		COOH	89		CONH <sub>2</sub>
73		COOH	100		COOH
74		COOH	102		COOH
75		COOH	111		
78		COOH			
85		CONH <sub>2</sub>			

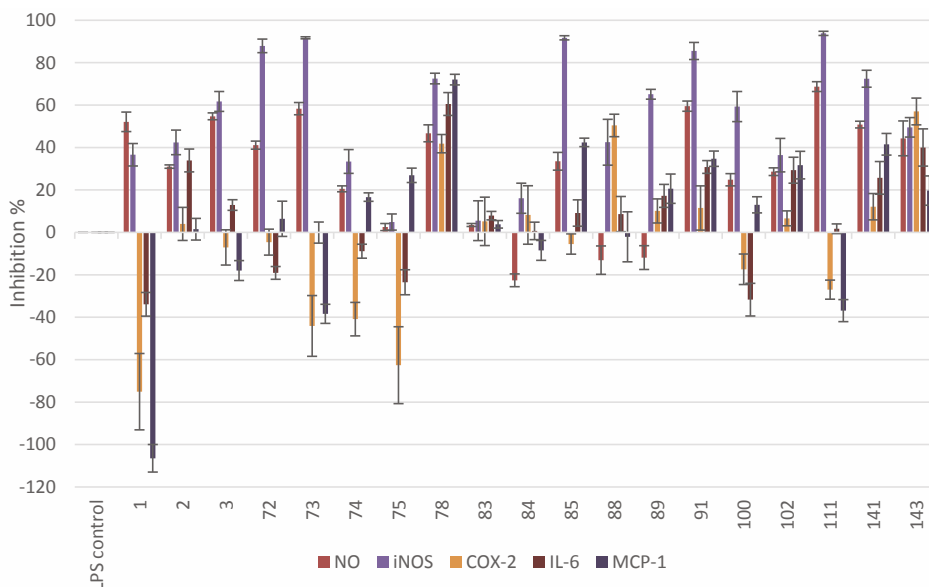
**Figure 52** Structures of the betulin derivatives tested for their ability to inhibit LPS-induced iNOS protein expression.<sup>IV</sup>



**Figure 53** Structures of the betulin derivatives tested for their ability to inhibit LPS-induced iNOS protein expression (cont'd).<sup>IV</sup>

The effects of the set of compounds on the expression of the inflammatory enzymes such as iNOS and COX-2, on the production of NO, interleukin-6 (IL-6), and chemotactic factor monocyte chemotactic protein-1 (MCP-1) were studied. The results are presented in Figure 54. Interestingly, some of the compounds, especially betulin (1), promoted the expression of inflammatory enzymes and the production of inflammation transmitters. However, compounds with inhibitory effects were found among the set as well.

Previously, ursolic acid (**5**) was shown to stimulate NO production, whereas its derivatives showed inhibitory activity.<sup>100</sup>

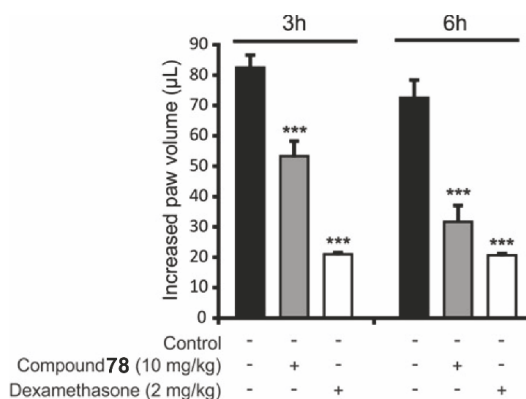


**Figure 54** Inhibiting effects of betulin derivatives on NO, iNOS, COX-2, IL-6, and MCP-1 in J774 macrophages.<sup>IV</sup>

Ten of the nineteen compounds tested showed over 50% inhibition of iNOS protein expression at 10  $\mu$ M concentration. Three of them (**73**, **85**, and **111**) decreased iNOS protein expression more than 90% at 10  $\mu$ M concentration, and they were selected for further dose-response studies. All of them gave clear dose-response effects, and their IC<sub>50</sub> values varied between 0.3-3  $\mu$ M. None of the compounds showed activity in iNOS mRNA assays, suggesting the effect of betulin derivatives on iNOS protein expression could be mediated through post-transcriptional mechanisms.

Three of the compounds (**78**, **88**, and **141**) inhibited COX-2 protein expression significantly at 10  $\mu$ M concentration. Compound **141** was the most potent COX-2 inhibitor with a 57% decrease of COX-2 protein expression. In LPS-induced NO production assays, five compounds (**1**, **3**, **73**, **91**, and **111**) stood out by reducing NO production over 50% at the 10  $\mu$ M concentration used.

Compound **78** was the most effective compound in both IL-6 production and MCP-1 production assays with 60% and 70% inhibitions, respectively. In further studies, it was shown that compound **78** suppresses iNOS and IL-6 mRNA levels significantly. These *in vitro* studies correlated with *in vivo* studies, as compound **78** significantly reduced carrageenan-induced paw inflammation in mice (Figure 55).



**Figure 55** The effect of the compound **78** on carrageenan-induced paw inflammation model in mice. Compound **78** (10 mg/kg) or dexamethasone (2 mg/kg) or saline were administered i.p. 2 h prior to carrageenan (1.5%) was injected into the paw. The edema was measured 3 h and 6 h after intraplantar injection and was compared to the basal level. Results are expressed as mean  $\pm$  SEM,  $n=6-12$ , \*\*\* $p<0.001$  as compared to mice without drug treatment. The contralateral control paw injected with saline developed no measurable edema.<sup>IV</sup>

The assayed betulin derivatives showed improved anti-inflammatory activity compared to natural triterpenoid betulin (**1**). The betulin derivatives **11**, **73**, **78**, and **85** showed inhibition of iNOS expression *in vitro*. The anti-inflammatory properties of compound **78** also correlated with those *in vivo*. To make further conclusions regarding the SARs, a wider and more diverse set of compounds should be tested. However, compound **78** could serve as a starting point for drug development.

## 6 Summary and conclusions

Betulin (**1**) is an abundant side product of the forestry industry without any meaningful use at the moment. Modifying betulin (**1**) chemically could increase its value as a fine chemical. In this work, betulin (**1**) was modified in order to produce suitable compounds for pharmaceutical applications. A set of fused heterocyclic derivatives of betulin was synthesized and SAR studies were conducted against leishmaniasis-causing protozoan parasites, ABHD12 serine hydrolase enzyme, prostate cancer cell lines and inflammation factors.

Among the tested heterocyclic compounds, inhibitory activity against *L. donovani* was shown. Compared to the previous results of our group<sup>9</sup>, some progress was made. However, the compounds produced were not active enough to justify further, more advanced stages of testing. If more potent betulin derivatives against *L. donovani* are to be found, further optimization work is needed.

ABHD12 was only discovered a few years ago and little is known about its structure. Due to the lack of a crystal structure, all information about its substrates are useful. Among the tested compounds, heterocyclic betulin derivatives were found to fully inhibit hABHD12 with IC<sub>50</sub> values in the submicromolar range. The compounds proved to be selective inhibitors towards ABHD12 without any effect on other endocannabinoid hydrolases or cannabinoid receptors. The first pharmacophore model for ABHD12 was developed, based on our SAR studies.

Some of the tested heterocyclic betulin derivatives showed anti-invasive activity in PC-3 cells, even at nanomolar concentrations, with minimal cytotoxicity. Our results show that betulin derivatives are a good starting point for discovery of more selective and less toxic treatments for metastatic cancers.

The fused heterocyclic derivatives of betulinic acid showed improved anti-inflammatory activity compared to the natural products betulin (**1**) or betulinic acid (**2**). The results were consistent *in vitro* and *in vivo*. Inflammation involves complex signaling cascades and many different mediators, which makes discovery of new anti-inflammatory compounds really challenging. In order to get SAR data and more potent anti-inflammatory agents, a wider set of betulin derivatives should be assayed. However, our compound **78** could work as a starting point for further development.

In this work, a number of new betulin derivatives was synthesized, and their biological activities with selected targets were tested. Some really good and unexpected bioactivities were found, even though more research is needed to get potent lead compounds. Other properties of the triterpenoids, such as lipophilicity and cytotoxicity, should also be taken in consideration when planning further development. One point proven herein is that natural products are still a valid starting point for modern drug discovery.

## References

1. Harvey, A.L., Edrada-Ebel, R. & Quinn, R.J. The re-emergence of natural products for drug discovery in the genomics era. *Nat. Rev. Drug Discov.* **14**, 111–129 (2015).
2. Newman, D.J. & Cragg, G.M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **75**, 311–335 (2012).
3. Sheng, H. & Sun, H. Synthesis, biology and clinical significance of pentacyclic triterpenes: a multi-target approach to prevention and treatment of metabolic and vascular diseases. *Nat. Prod. Rep.* **28**, 543–593 (2011).
4. Eckerman, C. & Ekman, R. Comparison of solvents for extraction and crystallization of betulinol from birch bark waste. *Pap. Puu* 100-106 (1985).
5. Pohjala, L., Alakurtti, S., Ahola, T., Yli-Kauhaluoma, J. & Tammela, P. Betulin-derived compounds as inhibitors of alphavirus replication. *J. Nat. Prod.* **72**, 1917–1926 (2009).
6. Kanamoto, T., Kashiwada, Y., Kanbara, K., Gotoh, K., Yoshimori, M. Goto, T., Sano, K. & Nakashima, H. Anti-human immunodeficiency virus activity of YK-FH312 (a betulinic acid derivative), a novel compound blocking viral maturation. *Antimicrob. Agents Chemother.* **45**, 1225-1230 (2001).
7. Mukherjee, P., Saha, K., Das, J., Pal, M. & Saha, B. Studies on the anti-inflammatory activity of rhizomes of *Nelumbo nucifera*. *Planta Med.* **63**, 367–369 (1997).
8. Steele, J.C.P., Warhurst, D.C., Kirby, G.C. & Simmonds, M.S. J. *In vitro* and *in vivo* evaluation of betulinic acid as an antimalarial. *Phytother. Res.* **13**, 115–119 (1999).
9. Alakurtti, S., Heiska, T., Kiriazis, A., Sacerdoti-Sierra, N., Jaffe, C.L. & Yli-Kauhaluoma, J. Synthesis and anti-leishmanial activity of heterocyclic betulin derivatives. *Bioorg. Med. Chem.* **18**, 1573–1582 (2010).
10. Pisha, E., Chai, H., Lee, I-S., Chagwedera T.E., Farnsworth, N.R., Cordell, G.A., Beecher, C.W.W., Fong, H.H.S., Kinghorn, A.D., Brown, D.M., Wani, M.C., Wall, M.E., Hieken, T.J., Das Gupta, T.K. & John M. Pezzuto, J.M. Discovery of betulinic

acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat. Med.* **1**, 1046–1051 (1995).

11. WHO Leishmaniasis. *WHO* (2015). at  
<<http://www.who.int/mediacentre/factsheets/fs375/en/>> (accessed 20.5.2015)
12. Sauvain, M., Kunesch, N., Poisson, J., Gantier, J-C., Gayral, P. & Dedet, J-P. Isolation of leishmanicidal triterpenes and lignans from the amazonian liana *Doliocarpus dentatus* (Dilleniaceae). *Phytother. Res.* **10**, 1–4 (1996).
13. Tan, N., Kaloga, M., Radtke, O.A., Kiderlen, A.F., Öksüz, S., Ulubelen, A. & Kolodziej, H. Abietane diterpenoids and triterpenoic acids from *Salvia cilicica* and their antileishmanial activities. *Phytochemistry* **61**, 881–884 (2002).
14. Torres-Santos, E.C., Lopes, D., Rodrigues Oliveira, R., Carauta, J.P.P., Bandeira Falcao, C.A., Kaplan, M.A.C. & Rossi-Bergmann B. Antileishmanial activity of isolated triterpenoids from *Pourouma guianensis*. *Phytomedicine* **11**, 114–120 (2004).
15. Sifaoui, I., López-Arencibia, A., Martín-Navarro, C.M., Ticona, J.C., Reyes-Batlle, M., Mejri, M., Jiménez, A.I., Lopez-Bazzocchi, I., Valladares, B., Lorenzo-Morales, J., Abderabba, M. & José E. Piñero, J.E. *In vitro* effects of triterpenic acids from olive leaf extracts on the mitochondrial membrane potential of promastigote stage of *Leishmania* spp. *Phytomedicine* **21**, 1689–1694 (2014).
16. Fokialakis, N., Kalpoutzakis, E., Tekwani, B.L., Skaltsounis, A.L. & Duke, S.O. Antileishmanial activity of natural diterpenes from *Cistus* sp. and semisynthetic derivatives thereof. *Biol. Pharm. Bull.* **29**, 1775–1778 (2006).
17. Ukil, A., Biswas, A., Das, T. & Das, P.K. 18 $\beta$ -Glycyrrhetic acid triggers curative Th1 response and nitric oxide up-regulation in experimental visceral leishmaniasis associated with the activation of NF- $\kappa$ B. *J. Immunol.* **175**, 1161–1169 (2005).
18. Bhattacharjee, A., Majumdera, S., Bhattacharyya Majumdera, S., Kumar Choudhurib, S., Royc, S. & Majumdar S. Co-administration of glycyrrhizic acid with



- the antileishmanial drug sodium antimony gluconate (SAG) cures SAG-resistant visceral leishmaniasis. *Int. J. Antimicrob. Agents* **45**, 268–277 (2015).
19. Dos Santos, V.A.F.F.M., Leite K.M., da Costa Siqueira, M., Regasini, L.O., Martinez, I., Nogueira, C.T., Kolos Galuppo, M., Stolf B.S., Soares Pereira, A.M., Cicarelli, R.M.B., Furlan, M. & Graminha, M.A.S. Antiprotozoal activity of quinonemethide triterpenes from *Maytenus ilicifolia* (Celastraceae). *Molecules* **18**, 1053–1062 (2013).
  20. Germonprez, N., Puyvelde, L.V., Maes, L., Tri, M.V. & Kimpe, N.D. New pentacyclic triterpene saponins with strong anti-leishmanial activity from the leaves of *Maesa balansae*. *Tetrahedron* **60**, 219–228 (2004).
  21. Delmas, F., Di Giorgio, C., Elias, R., Gasquet, M., Azas, N., Mshvildadze, V., Dekanosidze, G., Kemertelidze, E. & Timon-David, P. Antileishmanial activity of three saponins isolated from ivy,  $\alpha$ -hederin,  $\beta$ -hederin and hederacolchiside A1, as compared to their action on mammalian cells cultured *in vitro*. *Planta Med.* **66**, 343–347 (2000).
  22. Maes, L., Vanden Berghe, D., Germonprez, N., Quirijnen, L., Cos, P., De Kimpe, N. & Van Puyvelde, L. *In vitro* and *in vivo* activities of a triterpenoid saponin extract (PX-6518) from the plant *Maesa balansae* against visceral leishmania species. *Antimicrob. Agents Chemother.* **48**, 130–136 (2004).
  23. Germonprez, N., Maes, L., Van Puyvelde, L., Van Tri, M., Tuan, D.A., & De Kimpe, N. *In vitro* and *in vivo* anti-leishmanial activity of triterpenoid saponins isolated from *Maesa balansae* and some chemical derivatives. *J. Med. Chem.* **48**, 32–37 (2005).
  24. Maes, L., Germonprez, N., Quirijnen, L., Van Puyvelde, L., Cos, P. & Vanden Berghe, D. Comparative activities of the triterpene saponin maesabalide III and liposomal amphotericin B (AmBisome) against *Leishmania donovani* in hamsters. *Antimicrob. Agents Chemother.* **48**, 2056–2060 (2004).

25. Mandal, D., Panda, N., Kumar, S., Banerjee, S., Mandal, N.B. & Sahu, N.P. A triterpenoid saponin possessing antileishmanial activity from the leaves of *Careya arborea*. *Phytochemistry* **67**, 183–190 (2006).
26. Moiteiro, C., Marcelo Curto M.J., Mohamed N., Bailén M., Martínez-Díaz R. & González-Coloma A. Biovalorization of friedelane triterpenes derived from cork processing industry byproducts. *J. Agric. Food Chem.* **54**, 3566–3571 (2006).
27. Chowdhury, A., Mandal, S., Goswami A., Ghosh, M., Mandal, M. & Chakraborty, D. Dihydrobetulinic acid induces apoptosis in *Leishmania donovani* by targeting DNA topoisomerase I and II: implications in antileishmanial therapy. *Mol Med* **9**, 26–36 (2003).
28. Alakurtti, S., Bergstrom, P., Sacerdoti-Sierra, N., Jaffe, C.L. & Yli-Kauhaluoma, J. Anti-leishmanial activity of betulin derivatives. *J Antibiot* **63**, 123–126 (2010).
29. Chowdhury, S., Mukherjee, T., Sengupta, S., Chowdhury, S.R., Mukhopadhyay, S. & Majumder, H.K. Novel betulin derivatives as antileishmanial agents with mode of action targeting type IB DNA topoisomerase. *Mol. Pharmacol.* **80**, 694–703 (2011).
30. Domínguez-Carmona, D.B., Escalante-Erosa, F., García-Sosa, K., Ruiz-Pinell, G., Gutierrez-Yapu, D. & Chan-Bacab M.J. Antiprotozoal activity of betulinic acid derivatives. *Phytomedicine* **17**, 379–382 (2010).
31. Wert, L. Alakurtti, S., Corral, M.J., Sanchez-Fortun S., Yli-Kauhaluoma, J. & Alunda, J.M. Toxicity of betulin derivatives and *in vitro* effect on promastigotes and amastigotes of *Leishmania infantum* and *L. donovani*. *J. Antibiot.* **64**, 475–481 (2011).
32. Alcazar, W. López, A.S., Alakurtti, S., Tuononen, M-L., Yli-Kauhaluoma, J. & Ponte-Sucre, A. Betulin derivatives impair *Leishmania braziliensis* viability and host–parasite interaction. *Bioorg. Med. Chem.* **22**, 6220–6226 (2014).
33. Sousa, M.C. Varandas, R., Santos, R.C., Santos-Rosa, M., Alves, V. & Salvador, J.A.R. Antileishmanial activity of semisynthetic lupane triterpenoids betulin and

- betulinic acid derivatives: synergistic effects with miltefosine. *PLoS One* 9(3): e89939 (2014).
34. Morais, T. R.da Costa-Silva, T.A., Tempone, A.G., Borborema, S.E.T., Scotti, M.T., de Sousa, R.M.F., Araujo, A.C.C., de Oliveira, A., de Morais, S.A.L., Sartorelli, P. & Lago, J.H.G. Antiparasitic activity of natural and semi-synthetic tirucallane triterpenoids from *Schinus terebinthifolius* (Anacardiaceae): structure/activity relationships. *Molecules* **19**, 5761–5776 (2014).
  35. Mazoir, N. Benharref, A., Bailén, M., Reina, M., González-Coloma, A. & Martínez-Díaz, R.A. Antileishmanial and antitrypanosomal activity of triterpene derivatives from latex of two *Euphorbia* species. *Z. Naturforsch.* **66**, 360–366 (2011).
  36. Sheth, K., Jolad, S., Wiedhopf, R. & Cole, J.R. Tumor-inhibitory agent from *Hyptis emoryi* (Labiatae). *J. Pharm. Sci.* **61**, 1819–1819 (1972).
  37. Miles, D.H., Kokpol, U., Zalkow, L.H., Steindel, S. J. & Nabors, J.B. Tumor inhibitors I: Preliminary investigation of antitumor activity of *Sarracenia flava*. *J. Pharm. Sci.* **63**, 613–615 (1974).
  38. Trumbull, E.R., Bianchi, E., Eckert, D.J., Wiedhopf, R.M. & Cole, J.R. Tumor inhibitory agents from *Vauquelinia corymbosa* (rosaceae). *J. Pharm. Sci.* **65**, 1407–1408 (1976).
  39. Kim, J.Y., Koo, H.-M. & Kim, D. S.H.L. Development of C-20 modified betulinic acid derivatives as antitumor agents. *Bioorg. Med. Chem. Lett.* **11**, 2405–2408 (2001).
  40. Laszczyk, M. Pentacyclic triterpenes of the lupane, oleanane and ursane group as tools in cancer therapy. *Planta Med.* **75**, 1549–1560 (2009).
  41. Galgon, T., Wohlrab, W. & Dräger, B. Betulinic acid induces apoptosis in skin cancer cells and differentiation in normal human keratinocytes. *Exp. Dermatol.* **14**, 736–743 (2005).

42. Fulda, S. Friesen C, Los M, Scaffidi C, Mier W, Benedict M, Nuñez, G., Krammer, P.H., Peter, M.E. & Debatin, K.-M. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. *Cancer Res.* **57**, 4956–4964 (1997).
43. Tan, Y., Yu, R. & Pezzuto, J.M. Betulinic acid-induced programmed cell death in human melanoma cells involves mitogen-activated protein kinase activation. *Clin. Cancer Res.* **9**, 2866–2875 (2003).
44. Fulda, S. Betulinic acid: A natural product with anticancer activity. *Mol. Nutr. Food Res.* **53**, 140–146 (2009).
45. Mukherjee, R., Jaggi, M., Siddiqui, M.J.A., Srivastava, S.K., Rajendran, P., Vardhan, A. & Burman, A.C. Synthesis and cytotoxic activity of 3-*O*-acyl/3-hydrazine /2-bromo/20,29-dibromobetulinic acid derivatives. *Bioorg. Med. Chem. Lett.* **14**, 4087–4091 (2004).
46. Lee, S.-S., Chen, W.-C., Huang, C.-F. & Su, Y. Preparation and cytotoxic effect of ceanothic acid derivatives. *J. Nat. Prod.* **61**, 1343–1347 (1998).
47. Mukherjee, R. Jaggi, M., Rajendran, P., Siddiqui, M.J.A., Srivastava, S.K., Vardhan, A. & Burman, A.C. Betulinic acid and its derivatives as anti-angiogenic agents. *Bioorg. Med. Chem. Lett.* **14**, 2181–2184 (2004).
48. Šarek, J. Klinot, J., Džubák, P., Klinotová, E., Nosková. V., Křeček, V., Korínková, G., Thomson, J.O., Janost'áková, A., Wang, S., Parsons, S., Fischer, P.M., Zhelev, N.Z. & Hajdúch M. New lupane derived compounds with pro-apoptotic activity in cancer cells: Synthesis and structure–activity relationships. *J. Med. Chem.* **46**, 5402–5415 (2003).
49. Kim, D.S.H.L., Pezzuto, J.M. & Pisha, E. Synthesis of betulinic acid derivatives with activity against human melanoma. *Bioorg. Med. Chem. Lett.* **8**, 1707–1712 (1998).

50. Urban, M., Sarek, J., Klinot, J., Korinkova, G. & Hajduch, M. Synthesis of A-seco derivatives of betulinic acid with cytotoxic activity. *J. Nat. Prod.* **67**, 1100–1105 (2004).
51. Ma, C.-M., Cai, S.-Q., Cui, J.-R., Wang, R.-Q., Tu, P.-F., Hattori, M. & Daneshtalab, M. The cytotoxic activity of ursolic acid derivatives. *Eur. J. Med. Chem.* **40**, 582–589 (2005).
52. Kvasnica, M., Sarek, J., Klinotova, E., Dzubak, P. & Hajduch, M. Synthesis of phthalates of betulinic acid and betulin with cytotoxic activity. *Bioorg. Med. Chem.* **13**, 3447–3454 (2005).
53. Santos, R.C., Salvador, J.A.R., Marín, S., Cascante, M., Moreira, J.N. & Dinis, T.C.P. Synthesis and structure–activity relationship study of novel cytotoxic carbamate and N-acylheterocyclic bearing derivatives of betulin and betulinic acid. *Bioorg. Med. Chem.* **18**, 4385–4396 (2010).
54. Santos, R.C., Salvador, J.A.R., Marín, S. & Cascante, M. Novel semisynthetic derivatives of betulin and betulinic acid with cytotoxic activity. *Bioorg. Med. Chem.* **17**, 6241–6250 (2009).
55. Mar, A.A., Szotek, E.L., Koohang, A., Flavin, W.P., Eiznhamer, D.A., Flavin, M.T., Eiznhamer, D.A., Flavin, M.T. & Xu, Z.-Q. Synthesis, proapoptotic screening, and structure–activity relationships of novel aza-lupane triterpenoids. *Bioorg. Med. Chem. Lett.* **20**, 5389–5393 (2010).
56. Sharma, R. Guru, S.K., Jain, S.K., Pathania, A.S., Vishwakarma, R.A., Bhushan, S., Bhushan, S. & Bharate, S.B. 3-(2,6-Dichlorobenzyloxy)-11-oxo-olean-12-ene-29-oic acid, a semisynthetic derivative of glycyrrhetic acid: synthesis, antiproliferative, apoptotic and anti-angiogenesis activity. *Med. Chem. Commun.* **6**, 564–575 (2015).
57. Kommera, H., Kaluđerović, G.N., Dittrich, S., Kalbitz, J., Dräger, B., Mueller, T. & Paschke, R. Carbamate derivatives of betulinic acid and betulin with selective cytotoxic activity. *Bioorg. Med. Chem. Lett.* **20**, 3409–3412 (2010).

58. Kommera, H., Kaluđerović, G.N., Kalbitz, J. & Paschke, R. Synthesis and anticancer activity of novel betulinic acid and betulin derivatives. *Arch. Pharm.* **343**, 449–457 (2010).
59. Thibeault, D. Gauthier, C., Legault, J., Bouchard, J., Gagné, L. & Pichette, A. Synthesis and cytotoxicity of lupane-type triterpenoid glyceryl esters. *Bioorg. Med. Chem. Lett.* **22**, 4735–4739 (2012).
60. Csuk, R., Barthel, A., Schwarz, S., Kommera, H. & Paschke, R. Synthesis and biological evaluation of antitumor-active  $\gamma$ -butyrolactone substituted betulin derivatives. *Bioorg. Med. Chem.* **18**, 2549–2558 (2010).
61. Meng, Y.-Q., Liu, D., Cai L.-L., Chen, H., Cao, B. & Wang, Y.-Z. The synthesis of ursolic acid derivatives with cytotoxic activity and the investigation of their preliminary mechanism of action. *Bioorg. Med. Chem.* **17**, 848–854 (2009).
62. Bai, K.-K., Yu, Z., Chen, F.-L., Li, F., Li, W.-Y. & Guo, Y.-H. Synthesis and evaluation of ursolic acid derivatives as potent cytotoxic agents. *Bioorg. Med. Chem. Lett.* **22**, 2488–2493 (2012).
63. Sarek, J., Kvasnica, M., Urban, M., Klinot, J. & Hajduch, M. Correlation of cytotoxic activity of betulinines and their hydroxy analogues. *Bioorg. Med. Chem. Lett.* **15**, 4196–4200 (2005).
64. Urban, M., Vlk, M., Dzubak, P., Hajduch, M. & Sarek, J. Cytotoxic heterocyclic triterpenoids derived from betulin and betulinic acid. *Bioorg. Med. Chem.* **20**, 3666–3674 (2012).
65. Urban, M., Sarek, J., Kvasnica, M., Tislerova, I. & Hajduch, M. Triterpenoid pyrazines and benzopyrazines with cytotoxic activity. *J. Nat. Prod.* **70**, 526–532 (2007).
66. Gao, Y. Guo, X., Li, X., Liu, D., Song, D., Xu Y., Sun, M., Jing, Y. & Zhao, L. The Synthesis of glycyrrhetic acid derivatives containing a nitrogen heterocycle and

- their antiproliferative effects in human leukemia cells. *Molecules* **15**, 4439–4449 (2010).
67. Liby, K., Royce, D.B., Williams, C.R., Risingsong, R., Yore, M.M., Honda, T., Gribble, G.W., Dmitrovsky, E., Sporn, T.A. & Sporn, M.B. The synthetic triterpenoids CDDO-methyl ester and CDDO-ethyl amide prevent lung cancer induced by vinyl carbamate in A/J mice. *Cancer Res.* **67**, 2414–2419 (2007).
  68. Suh, N., Wang, Y., Honda, T., Gribble, G.W., Dmitrovsky, E., Hickey, W.F., Robert A. Maue, R.A., Place, A.E., Porter, D.M., Spinella, M.J., Charlotte R. Williams, C.R., Gengfei Wu, G., Dannenberg, A.J., Flanders, K.C., Letterio, J.J., Mangelsdorf, D.J. Nathan, C.F., Nguyen, L., Porter, W.W., Ren, R.F., Roberts, A.B., Roche, N.S., Subbaramaiah, K., & Sporn, M.B. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. *Cancer Res.* **59**, 336–341 (1999).
  69. Zou, W., Chen, S., Liu, X., Yue, P., Sporn, M. B., Khuri, F. R. & Sun, S-Y. c-FLIP downregulation contributes to apoptosis induction by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me) in human lung cancer cells. *Cancer Biol. Ther.* **6**, 1614–1620 (2007).
  70. Reata Pharmaceuticals, Inc. RTA 402 in Patients With Advanced Solid Tumors or Lymphoid Malignancies In ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2007-2014 [cited 2015 Sept 8] Available from: <https://clinicaltrials.gov/ct2/show/NCT00529438?term=CDDO-Me&rank=4> NML Identifier: NCT00529438
  71. You, Y.-J., Kim, Y., Nam, N.-H. & Ahn, B.-Z. Synthesis and cytotoxic activity of A-ring modified betulinic acid derivatives. *Bioorg. Med. Chem. Lett.* **13**, 3137–3140 (2003).

72. Csuk, R., Stark, S., Nitsche, C., Barthel, A. & Siewert, B. Alkylidene branched lupane derivatives: Synthesis and antitumor activity. *Eur. J. Med. Chem.* **53**, 337–345 (2012).
73. Chatterjee, P., Pezzuto, J. M. & Kouzi, S. A. Glucosidation of betulinic acid by *Cunninghamella* species. *J. Nat. Prod.* **62**, 761–763 (1999).
74. Csuk, R., Barthel, A., Kluge, R. & Ströhl, D. Synthesis, cytotoxicity and liposome preparation of 28-acetylenic betulin derivatives. *Bioorg. Med. Chem.* **18**, 7252–7259 (2010).
75. Hata, K., Hori, K. & Takahashi, S. Differentiation- and apoptosis-inducing activities by pentacyclic triterpenes on a mouse melanoma cell line. *J. Nat. Prod.* **65**, 645–648 (2002).
76. Csuk, R., Barthel, A., Kluge, R., Ströhl, D., Kommera, H. & Paschke, R. Synthesis and biological evaluation of antitumour-active betulin derivatives. *Bioorg. Med. Chem.* **18**, 1344–1355 (2010).
77. Dang Thi, T.A., Kim Tuyet, N.T., Pham The, C., Thanh Nguyen, H., Ba Thi, C., Doan Duy T., D’hooghe, M. & Van Nguyen, T. Synthesis and cytotoxic evaluation of novel ester-triazole-linked triterpenoid–AZT conjugates. *Bioorg. Med. Chem. Lett.* **24**, 5190–5194 (2014).
78. Dang Thi, T.A., Kim Tuyet, N.T., Pham The, C., Thanh Nguyen, H., Ba Thi, C., Thi Phuong, H., Van Boi, L., Van Nguyen, T. & D’hooghe, M. Synthesis and cytotoxic evaluation of novel amide-triazole-linked triterpenoid–AZT conjugates. *Tetrahedron Lett.* **56**, 218–224 (2015).
79. Csuk, R., Barthel, A., Sczepek, R., Siewert, B. & Schwarz, S. Synthesis, encapsulation and antitumor activity of new betulin derivatives. *Arch. Pharm.* **344**, 37–49 (2011).



80. Gauthier, C., Legault, J., Lavoie, S., Rondeau, S., Tremblay, S. & Pichette A. Synthesis and cytotoxicity of bidesmosidic betulin and betulinic acid saponins. *J. Nat. Prod.* **72**, 72–81 (2009).
81. Sidoryk, K., Korda, A., Rárová, L., Oklešťková, J., Strnad, M., Cmoch, P., Pakulski, Z., Gwardiak, K., Karczewski, R. & Luboradzki, R. Synthesis and biological activity of new homolupanes and homolupane saponins. *Tetrahedron* **71**, 2004–2012 (2015).
82. Boryczka, S., Bębenek, E., Wietrzyk, J., Kempieńska, K., Jastrzębska, M., Kusz, J. & Nowak, M. Synthesis, structure and cytotoxic activity of new acetylenic derivatives of betulin. *Molecules* **18**, 4526–4543 (2013).
83. Csuk, R., Szczepek, R., Siewert, B. & Nitsche, C. Cytotoxic betulin-derived hydroxypropargylamines trigger apoptosis. *Bioorg. Med. Chem.* **21**, 425–435 (2013).
84. Urban, M., Sarek, J., Tislerova, I., Dzubak, P. & Hajduch, M. Influence of esterification and modification of A-ring in a group of lupane acids on their cytotoxicity. *Bioorg. Med. Chem.* **13**, 5527–5535 (2005).
85. Jeong, H.-J., Chai, H.-B., Park, S.-Y. & Kim, D.S.H.L. Preparation of amino acid conjugates of betulinic acid with activity against human melanoma. *Bioorg. Med. Chem. Lett.* **9**, 1201–1204 (1999).
86. Liby, K.T., Yore, M.M. & Sporn, M.B. Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer. *Nat. Rev. Cancer* **7**, 357–369 (2007).
87. Patlolla, J.M.R. & Rao, C.V. Triterpenoids for cancer prevention and treatment: current status and future prospects. *Curr. Pharm. Biotechnol.* **13**, 147–155 (2012).
88. Braca, A., Dal Piaz, F., Marzocco, S., Autore, G., Vassallo, A., De Tommasi, N. Triterpene derivatives as inhibitors of protein involved in the inflammatory process: molecules interfering with phospholipase A2, cyclooxygenase, and lipoxygenase. *Curr. Drug Targets* **12**, 302–321 (2011).

89. Mantovani, A. & Pierotti, M.A. Cancer and inflammation: A complex relationship. *Cancer Lett.* **267**, 180–181 (2008).
90. Simonian, N.A. & Coyle, J.T. Oxidative stress in neurodegenerative diseases. *Annu. Rev. Pharmacol. Toxicol.* **36**, 83–106 (1996).
91. Misko, T.P., Trotter, J.L. & Cross, A.H. Mediation of inflammation by encephalitogenic cells: Interferon  $\gamma$  induction of nitric oxide synthase and cyclooxygenase 2. *J. Neuroimmunol.* **61**, 195–204 (1995).
92. Crofford, L.J., Wilder, R.L., Ristimäki, A.P., Sano, H., Remmers, E.F., Epps, H.R. & Hla, T. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. *J. Clin. Invest.* **93**, 1095–1101 (1994).
93. Sano, H., Kawahito, Y., Wilderm R.L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, s., Kato, H., Kondo, M., & Hla, T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res.* **55**, 3785–3789 (1995).
94. Yasukawa, K., Takido, M., Takeuchi, M. & Nakagawa, S. Effect of chemical constituents from plants on 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation in mice. *Chem. Pharm. Bull. (Tokyo)* **37**, 1071–1073 (1989).
95. Yasukawa, K., Takido, M., Matsumoto, T., Takeuchi, M. & Nakagawa, S. Sterol and triterpene derivatives from plants inhibit the effects of a tumor promoter, and sitosterol and betulinic acid inhibit tumor formation in mouse skin two-stage carcinogenesis. *Oncology* **48**, 72–76 (1991).
96. Singh, G.B., Singh, S., Bani, S., Gupta, B.D. & Banerjee, S.K. Anti-inflammatory activity of oleanolic acid in rats and mice. *J. Pharm. Pharmacol.* **44**, 456–458 (1992).
97. Wu, Q., Li, H., Qiu, J. & Feng, H. Betulin protects mice from bacterial pneumonia and acute lung injury. *Microb. Pathog.* **75**, 21–28 (2014).

98. Del Carmen Recio, M., Giner, R., Máñez, S. & Ríos, J. Structural requirements for the anti-inflammatory activity of natural triterpenoids. *Planta Med.* **61**, 182–185 (1995).
99. Soldi, C., Pizzolatti, M.G., Luiz, A.P., Marcon, R., Meotti, F.C., Miotto, L.A. & Santos, A.R.S. Synthetic derivatives of the  $\alpha$ - and  $\beta$ -amyrin triterpenes and their antinociceptive properties. *Bioorg. Med. Chem.* **16**, 3377–3386 (2008).
100. Honda, T., Finlay, H. J., Gribble, G.W., Suh, N. & Sporn, M.B. New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **7**, 1623–1628 (1997).
101. Suh, N., Honda, T., Finlay, H.J., Barchowsky, A., Williams, C., Benoit, N.E., Xie, Q., Nathan, C., Gribble, G.W. & Sporn, M.B. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. *Cancer Res.* **58**, 717–723 (1998).
102. Honda, T., Rounds, B.V., Gribble, G.W., Suh, N., Wang, Y. & Sporn, M.B. Design and synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **8**, 2711–2714 (1998).
103. Wu, T., Ye, Y., Min, S.-Y., Zhu, J., Khobahy, E., Zhou, J., Yan, M., Hemachandran, S., Pathak, S., Zhou, X.J., Andreeff, M. & Mohan C. Prevention of murine lupus nephritis by targeting multiple signaling axes and oxidative stress using a synthetic triterpenoid. *Arthritis Rheumatol.* **66**, 3129–3139 (2014).
104. Sporn, M.B., Liby, K.T., Yore, M.M., Fu, L., Lopchuk, J.M. & Gribble, G.W. New synthetic triterpenoids: Potent agents for prevention and treatment of tissue injury caused by inflammatory and oxidative stress. *J. Nat. Prod.* **74**, 537–545 (2011).
105. Bednarczyk-Cwynar, B., Zaprutko, L., Marciniak, J., Lewandowski, G., Szulc, M., Kaminska, E., Wachowiak, N. & Mikolajczak, P.L. The analgesic and anti-

- inflammatory effect of new oleanolic acid acyloxyimino derivative. *Eur. J. Pharm. Sci.* **47**, 549–555 (2012).
106. Mueller, D., Triebel, S., Rudakovski, O. & Richling, E. Influence of triterpenoids present in apple peel on inflammatory gene expression associated with inflammatory bowel disease (IBD). *Food Chem.* **139**, 339–346 (2013).
  107. Choi, S., Nguyen, V.T., Tae, N., Lee, S., Ryoo, S., Min, B.-S. & Lee, J.-H. Anti-inflammatory and heme oxygenase-1 inducing activities of lanostane triterpenes isolated from mushroom *Ganoderma lucidum* in RAW264.7 cells. *Toxicol. Appl. Pharmacol.* **280**, 434–442 (2014).
  108. Fabri, R.L., Garcia, R.A., Florêncio, J.R., de Carvalho, L.O., de Pinto, N.C.C., Coimbra E.S., de Souza-Fagundes, E.M., Ribeiro, A. & Scio, E. Pentacyclic triterpenoids from *Mitracarpus frigidus* (Willd. ex Roem. & Schult.) K. Shum: *in vitro* cytotoxic and leishmanicidal and *in vivo* anti-inflammatory and antioxidative activities. *Med. Chem. Res.* **23**, 5294–5304 (2014).
  109. Govdi, A.I., Sokolova, N.V., Sorokina, I.V., Baev, D.S., Tolstikova, T.G., Mamatyuk, V.I., Fadeev, D.S., Vasilevsky, S.F. & Nenajdenko, V.G. Synthesis of new betulinic acid–peptide conjugates and *in vivo* and *in silico* studies of the influence of peptide moieties on the triterpenoid core activity. *Med. Chem. Commun.* **6**, 230–238 (2014).
  110. Hui, B., Yao, X., Zhou, Q., Wu, Z., Sheng, P. & Zhang, L. Pristimerin, a natural anti-tumor triterpenoid, inhibits LPS-induced TNF- $\alpha$  and IL-8 production through down-regulation of ROS-related classical NF- $\kappa$ B pathway in THP-1 cells. *Int. Immunopharmacol.* **21**, 501–508 (2014).
  111. Li, M.-M., Su, X.-Q., Sun, J., Gu, Y.-F., Huang, Z., Zeng, K.-W., Zhang, Q., Zhao, Y.-F., Ferreira, D., Zjawiony, J.K., Li, J. & Tu, P.-F. Anti-inflammatory ursane- and oleanane-type triterpenoids from *Vitex negundo* var. *cannabifolia*. *J. Nat. Prod.* **77**, 2248–2254 (2014).

112. Verhoff, M., Seitz, S., Paul, M., Noha, S.M., Jauch, J., Schuster, D. & Werz, O. Tetra- and pentacyclic triterpene acids from the ancient anti-inflammatory remedy frankincense as inhibitors of microsomal prostaglandin E2 synthase-1. *J. Nat. Prod.* **77**, 1445–1451 (2014).
113. Hsu, C.-L., Fang, S.-C., Huang, H.-W. & Yen, G.-C. Anti-inflammatory effects of triterpenes and steroid compounds isolated from the stem bark of *Hiptage benghalensis*. *J. Funct. Foods* **12**, 420–427 (2015).
114. Pacher, P., Bátkai, S. & Kunos, G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol. Rev.* **58**, 389–462 (2006).
115. Simão da Silva, K.A.B., Paszcuk, A.F., Passos, G.F., Silva, E.S., Bento, A.F., Meotti, F.C. & Calixto, J.B. Activation of cannabinoid receptors by the pentacyclic triterpene  $\alpha,\beta$ -amyrin inhibits inflammatory and neuropathic persistent pain in mice. *PAIN* **152**, 1872–1887 (2011).
116. Di Marzo, V. Targeting the endocannabinoid system: to enhance or reduce? *Nat. Rev. Drug Discov.* **7**, 438–455 (2008).
117. Liu, X., Jutooru, I., Lei, P., Kim, K., Lee, S., Brents, L.K., Prather, P.L. & Safe, S. Betulinic acid targets YY1 and ErbB2 through cannabinoid receptor-dependent disruption of microRNA-27a:ZBTB10 in breast cancer. *Mol. Cancer Ther.* **11**, 1421–1431 (2012).
118. Matos, I., Bento, A.F., Marcon, R., Claudino, R.F. & Calixto, J.B. Preventive and therapeutic oral administration of the pentacyclic triterpene  $\alpha,\beta$ -amyrin ameliorates dextran sulfate sodium-induced colitis in mice: The relevance of cannabinoid system. *Mol. Immunol.* **54**, 482–492 (2013).
119. Dutra, R.C., Simão da Silva, K.A.B., Bento, A.F., Marcon, R., Paszcuk, A.F., Meotti, F.C., Pianowski, L.F. & Calixto, J.B. Euphol, a tetracyclic triterpene produces antinociceptive effects in inflammatory and neuropathic pain: The involvement of cannabinoid system. *Neuropharmacology* **63**, 593–605 (2012).

120. King, A.R., Dotsey, E.Y., Lodola, A., Jung, K.M., Ghomian, A., Qiu, Y., Fu, J., Mor, M. & Piomelli, D. Discovery of potent and reversible monoacylglycerol lipase inhibitors. *Chem. Biol.* **16**, 1045–1052 (2009).
121. Chicca, A., Marazzi, J. & Gertsch, J. The antinociceptive triterpene  $\beta$ -amyrin inhibits 2-arachidonoylglycerol (2-AG) hydrolysis without directly targeting cannabinoid receptors. *Br. J. Pharmacol.* **167**, 1596–1608 (2012).
123. Callahan, H. L., Portal, A. C., Devereaux, R. & Grogl, M. An axenic amastigote system for drug screening. *Antimicrob. Agents Chemother.* **41**, 818–822 (1997).
122. Pichette, A., Liu, H., Roy C., Tanguay, S., Simard, F. & Lavoie S. Selective oxidation of betulin for the preparation of betulinic acid, an antitumoral compound. *Synth. Commun.* **34**, 3925–3937 (2004).
123. Melnikova, N., Burlova, I., Kiseleva, T., Klabukova, I., Gulenova, M., Kislitsin, A., Vasin, V. & Tanaseichuk, B.A Practical synthesis of betulonic acid using selective oxidation of betulin on aluminium solid support. *Molecules* **17**, 11849–11863 (2012).
124. Tulisalo, J., Pirttimaa, M., Alakurtti, S., Yli-Kauhaluoma, J. & Koskimies, S. Method for preparation of betulinic acid. (2013). at [http://worldwide.espacenet.com/publicationDetails/biblio?FT=D&date=20130321&DB=EPODOC&locale=en\\_EP&CC=WO&NR=2013038314A1&KC=A1&ND=4](http://worldwide.espacenet.com/publicationDetails/biblio?FT=D&date=20130321&DB=EPODOC&locale=en_EP&CC=WO&NR=2013038314A1&KC=A1&ND=4)
125. Schulze, H. & Pieroh, K. Zur kenntnis des betulins. *Dtsch. Chem. Ges. B Ser.* **55**, 2332–2346 (1922).
126. Blankman, J.L., Simon, G.M. & Cravatt, B.F.A Comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem. Biol.* **14**, 1347–1356 (2007).
127. Fiskerstrand, T., H'mida-Ben Brahim, D., Johansson, S., M'zahem, A., Haukanes, B.I., Drouot N., Zimmermann, J., Cole, A.J., Vedeler, C., Bredrup, C., Assoum, M., Tazir, M., Klockgether, T., Hamri, A., Steen,V.M. Boman, H., Bindoff, L.A., Koenig, M. & Knappskog, P.M. Mutations in ABHD12 cause the neurodegenerative

- disease PHARC: An inborn error of endocannabinoid metabolism. *Am. J. Hum. Genet.* **87**, 410–417 (2010).
128. Laitinen, T., Navia-Paldanius, D., Ryttilahti, R., Marjamaa, J.J.T., Kařizková, J., Parkkari, T., Pantsar, T., Poso, A., Laitinen, J.T. & Savinainen, J.R. Mutation of cys242 of human monoacylglycerol lipase disrupts balanced hydrolysis of 1- and 2-monoacylglycerols and selectively impairs inhibitor potency. *Mol. Pharmacol.* **85**, 510–519 (2014).
129. Das, S., Addis, D., Zhou, S., Junge, K. & Beller, M. Zinc-catalyzed reduction of amides: Unprecedented selectivity and functional group tolerance. *J. Am. Chem. Soc.* **132**, 1770–1771 (2010).
130. Adibekian, A., Martin, B.R., Wang, C., Hsu, K.-L., Bachovchin, D.A., Niessen, S., Hoover, H. & Cravatt, B.F. Click-generated triazole ureas as ultrapotent *in vivo*-active serine hydrolase inhibitors. *Nat. Chem. Biol.* **7**, 469–478 (2011).
131. Hao, J., Zhang, P., Wen, X. & Sun, H. Efficient access to 2-isobetulinic acid, 2-isoleanolic acid, and 2-isoursolic acid. *J. Org. Chem.* **73**, 7405–7408 (2008).
132. Rzeski, W., Stepulak, A., Szymański, M., Juszczak, M., Grabarska, A., Sifringer, M., Kaczor, J. & Kandefer-Szerszen, M. Betulin elicits anti-cancer effects in tumour primary cultures and cell lines *in vitro*. *Basic Clin. Pharmacol. Toxicol.* **105**, 425–432 (2009).

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